DETERMINING FACE, PREDICTIVE, CONSTRUCT VALIDITY AND NOVEL RECEPTOR TARGETS IN A SPONTANEOUS COMPULSIVE-LIKE MOUSE MODEL

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Abstract

Obsessive-compulsive disorder (OCD) is one of the most prevalent neuropsychiatric disorders with no known etiology. Genetic variation, sex differences and physiological stages, such as pregnancy, postpartum and menopause in females, are important factors that are thought to modulate the pathophysiology of the disorder. Deeper understanding of these factors and their role in modulating behaviors is essential to unraveling the complex clinical heterogeneity of OCD. Using a novel mouse model that exhibits a spontaneous compulsive-like phenotype, I investigated the role of strain differences, sex differences, ovarian sex hormones and postpartum lactation in influencing compulsive-like and affective behaviors. Due to the lack of definite neural substrates and first line therapeutic options for treatment resistant patients, I also probed into the role of positive allosteric modulation of nicotinic acetylcholine receptor subtype as a therapeutic target for translational prospects. The thesis showed several significant and novel findings. Strain and sex comparisons of the compulsive-like mouse strains (BIG1 and BIG2) showed that the behavioral outcomes and HPA axis response can be influenced by sex, genotype and sex by genotype interactions. Acute ovariectomy and behavioral assessments after one week, which mimics surgical menopause in humans, increased the compulsive-like behaviors in the BIG strains only. Acute ovariectomy also exacerbated anxiety-like behaviors in the compulsive-like BIG strains. This exacerbation of compulsive-like behaviors was restored only by estrogen and not progesterone, while estrogen and progesterone both restored anxiety-like behaviors based on the strain and the type of behavioral assessments. Lactating postpartum compulsive-like female mice were protected against compulsive-like behaviors and showed enhanced responsiveness to selective serotonin reuptake inhibitors (SSRIs) compared to the non-lactating and the nulliparous females. Finally, acute and chronic administration of desformylflustrabromine (dFBr), a positive allosteric modulator of α4β2 nicotinic acetylcholine receptors, resulted in the attenuation of compulsive-like behaviors in the mouse model, while

not affecting anxiety-like behaviors. The current thesis work therefore provides a foundation for further exploration of factors like strain, sex, physiological status and cholinergic receptor subtypes in mouse models to understand the neurobiology and behavioral correlates of OCD in humans.

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Chapter 1: Overview and Research Aims

1.1 Background

1.1.1 Obsessive-Compulsive Disorder

Obsessive-compulsive disorder (OCD) is a frequently occurring neuropsychiatric disorder that impacts almost 2% of the US population (Ruscio et al., 2010) and has a life time prevalence of 1-3% making it the fourth most common psychiatric illness (Karno et al., 1988; Ruscio et al., 2010). It was first described as a psychiatric condition about 100 years ago (Janet and Raymond, 1903). The world health organization has indicated OCD to be the leading global cause of non-fatal illness (Ayuso-Mateos, 2006). Previously, OCD has been considered among the top 20 causes of years of life spent with disability for patients between 15-44 years old (Murray and Lopez, 1996). It has also been estimated that individuals with OCD spend an average of 17 years before being diagnosed and treated (Hollander and Weilgus-Kornwasser, 1997). This is mainly because patients are inclined to conceal their symptoms fearing they will appear crazy and seek medical attention only when other co-morbid conditions like anxiety and depression exacerbate the disorder (Abramowitz et al., 2009; Fullana et al., 2009). Ineffective identification of specific symptoms is also one of the reasons for delayed diagnosis (Marazziti et al., 2012).

Symptomatically, OCD is characterized by unwanted intrusive thoughts (obsessions) and repetitive behaviors (compulsions). Obsessions can be termed as an idea or impulse which can be characterized as recurrent, forceful and persistent that does not disappear despite attempts to ignore or suppress it. Compulsions on the other hand, are repetitive, perseverant and over indulgent acts in response to the obsessions (American Psychiatric Association, 2013; Grant, 2014). Compulsions are typically meant to neutralize the obsessive feelings (Grant, 2014), but provide only transient relief and lead to reinforcement of the behaviors and

continuation of the obsession compulsion cycle (Pauls et al., 2014). This significantly impacts human and social functioning, socio-economic status, family relationships and general quality of life (Fontenelle et al., 2010; Hollander et al., 2010). The age of onset ranges from early in childhood to adulthood (Pauls et al., 2014). Onset after 30 years of age is highly unusual (Jenike, 2004; Ruscio et al., 2010). Approximately 30-50% of the patients have onset of symptoms before 10 years of age indicating neurodevelopmental aspects to the disorder (Geller et al., 1998; Zohar, 1999; Nakatani et al., 2011). Interestingly, in childhood onset OCD, males are more commonly affected than females with a ratio of approximately 2:1 to 3:1 (males: females; Kalra and Swedo, 2009). This ratio however shifts to 1:1.4 among patients with onset during or after puberty. Though genetic linkage, candidate gene and genome wide association studies in humans (Goodman et al., 1990; Abelson et al., 2005; Shugart et al., 2006; Samuels et al., 2007; Hanna et al., 2007; Ross et al., 2011; Mathews et al., 2012; Stewart et al., 2013; Taylor., 2013) have indicated many genetic associations, the actual causative factor has been elusive (Grant, 2014). This could be attributed to the heterogeneous nature of the disorder.

1.1.2 Clinical Heterogeneity of OCD

While obsessions and compulsions are cardinal characteristics of OCD, the specific content of these obsessions and compulsions vary widely (Murphy et al., 2010). For example, contamination obsessions and subsequent cleaning compulsions are a common type of OCD symptoms, but the clinical expression of the contamination fear is broad (Pauls et al., 2014). Contamination fears or disgust can range from germs, dirt, events, place or an experience. On the other hand, some patients experience obsessions-only symptoms without any compulsive ritualistic behaviors. An example is that of fear of causing harm to others or self (Pauls et al., 2014). Many studies use the Yale-Brown Obsessive Compulsive Scale (Y-BOCS scale) to clinically characterize and identify the occurrence and types of obsessive-compulsive symptoms (Goodman et al., 1989). The Y-BOCS is an ordinal scale that has a rating of 0-4 to score the

clinical severity with 0 being the least time spent on obsessions and compulsions and 4 being the most time spent (Pauls et al., 2014). Further enhancements to this scale involve the Dimensional Yale-Brown Obsessive Compulsive Scale (DY-BOCS) which categorizes OCD into 6 distinct dimensions (Rosario-Campos et al., 2006). They are i) aggression/checking, ii) sexual/moral/religious, iii) symmetry/ordering/counting, iv) contamination/washing, v) hoarding and vi) others (Rosario-Campos et al., 2006; Pauls et al., 2014; Okada et al., 2015). In addition, researchers have divided OCD into subgroups based on familiality, gender, age of onset and comorbid patterns (Hasler et al., 2005; Miguel et al., 2005; Mataix-cols and van den Heuvel, 2006; Catapano et al., 2010).

The most common comorbid disorders associated with OCD are depression and anxiety (Overbeek et al., 2002; Tukel et al., 2002; Torres et al., 2006; Boileau, 2011). Other less frequently observed comorbid symptoms include eating disorders, alcohol abuse, tricothillomania (compulsive hair pulling), body dysmorphic disorder, bipolar disorder and Tourette syndrome (Ferraro et al., 2009; Mancebo et al., 2009; D'Ambrosio et al., 2010; Murphy et al., 2010; Tyagi et al., 2015). Previously, OCD was categorized as an anxiety disorder in the DSM-III and DSM-IV manuals (American Psychiatric Association, 1994). However, the current version of DSM (DSM-V) declassifies OCD from anxiety disorders. DSM-V designates a new category called the OCRD (obsessive-compulsive related disorders) which includes OCD, body dysmorphic disorder, hoarding, trichothillomania, excoriation, substance/medication-induced OCD and related disorders (American Psychiatric Association, 2013).

1.1.3 Etiology and Pathophysiology of OCD

1.1.3.1 Environmental and Genetic Factors

Like many other psychiatric disorders, environmental and genetic factors contribute to the etiology of OCD. A study (Hudziak et al., 2004) in 7-12 year old twins indicates additive genetic factors as the reason for 47-58% of the variance in obsessive-compulsive behaviors. The remaining variance is attributed to environmental influences. According to a study (Cath et al., 2008), environmental factors explain nearly half of the symptom persistence in boys and two third in girls. Some of the critical environmental factors contributing to the obsessive-compulsive phenomenology are low socio-economic status, over protected or neglected childhood (Cavedo and Parker, 1994) and lack of parental emotional warmth (Alonso et al., 2004). Childhood sexual abuse has also been shown to mediate OCD especially in women (Lochner et al., 2002). The role of religious beliefs on display and exacerbation of obsessions and compulsions has been controversial. Some studies have found a positive correlation (Abramowitz et al., 2004) while others have not (Assarian et al., 2006). In addition, perinatal risk factors such as extended labor and edema during pregnancy can increase the onset probability of the disorder (Vasconcelos et al., 2007). Other notable environmental factors include streptococcus infections, which exacerbate obsessive-compulsive symptoms (Boileau, 2011).

Family studies, segregation analysis studies, candidate gene studies and genome wide association studies have consistently reported and established heritability and genetic mechanisms as one of the important causative factors of OCD. Family studies have conclusively shown that early onset OCD has a familial basis (Pauls et al., 1995; Nestadt et al., 2000). Family studies with adult probands have shown that OCD is familial with increased risk of occurrence among relatives of patients with childhood onset OCD (Fyer et al., 2005; Grabe et al., 2006; Black et al., 2013). Around 100 candidate gene studies have focused on the genetic variants predominantly within neurotransmitter pathways (Pauls et al., 2014). One of these

candidate gene studies has shown that OCD can be associated with polymorphisms in serotonin system related genes such as 5HTTLPR (serotonin-transporter linked polymorphic region) and HTR2A (5-hydroxytryptophan receptor 2A) (Taylor et al., 2013). The same study also pinpointed genetic variations in monoamine oxidase and catechol-o methyl transferase genes only in male patients and dopamine system related genes like DAT-1 (Dopamine active transporter 1 gene) and DRD3 (Dopamine receptor D3) (Taylor et al., 2013). In addition, other genome wide association studies have found various genes that code for essential neurobiological substrates (Schweitzer et al., 2002; Perez-Torrado et al., 2006; Wieczorek et al., 2010; Stewart et al., 2013), which are essential for neuronal transmission and plasticity, such as DLGAP1 (guanylate kinase-associated protein), BTBD3 (BTB domain containing protein 3) etc.

1.1.3.2 Neural Basis: Circuits and Function

A substantial number of neuroimaging, pharmacological and neuropsychological studies have facilitated a better understanding of the neural substrates and circuitry implicated in OCD (Pauls et al., 2014). One of the most prevailing neurocircuitry models thought to account for the pathophysiology of OCD has been the cortico-striato-thalamo-cortico (CSTC) circuit model first proposed by Saxena and Rauch (Saxena and Rauch, 2000). The fronto-striatal circuitry comprises of direct and indirect pathways. In normal individuals, the excitatory direct pathway is counteracted by the inhibitory indirect pathway forming a dynamic balance (Pauls et al., 2014). In OCD patients, there is dysregulation of this pathway leading to hyperactivation of the direct orbitofrontal-subcortical pathway (Saxena and Rauch, 2000). This seminal finding by Saxena and Rauch is based on prior studies that have shown increased activity in orbitofrontal cortex, caudate nucleus, globus pallidus and dorsal nucleus of the thalamus of OCD patients (Hoehn-Saric et al., 1991; Schwartz et al., 1996; Saxena et al., 1998). Further support for the CSTC hypothesis has come from functional connectivity studies that indicate abnormal prefrontal and striatal regions in patients (Harrison et al., 2009; Fitzgerald et al., 2011). Moreover, proton

magnetic resonance spectroscopy studies (Brennan et al., 2013), optogenetic studies in animal models (Ahmari et al., 2013; Burguiere et al., 2013) and pharmacological treatment studies (Saxena et al., 2009; Freyer et al., 2011) have also corroborated the CSTC model. Neuropsychological studies have however provided inconclusive evidence on the target brain region (Kuelz et al., 2004; Abramovitch et al., 2012; Abramovitch et al., 2013). One of the outcomes of these neuropsychological studies is that, neural circuitries differ among OCD patients with symptom dimensions and therefore could have distinct and overlapping neural correlates (Van Den Heuvel et al., 2005).

1.1.3.3 Neurochemistry of OCD

Several converging lines of evidence point towards neurobiological mechanisms that contribute towards the etiology of OCD. Pharmacological and neurobiological studies on both humans and animal models have implicated central neurotransmitters, peptide and steroid systems in the pathophysiology of OCD (Pauls et al., 2010). Some of them are as follows:

i) Serotonergic hypothesis: Serotonin (5-HT) is a monoamine neurotransmitter biochemically derived from the amino acid tryptophan (González-Flores et al., 2011). The B1-B9 neurons of the raphe nuclei in the brainstem are the principal source of serotonin in the brain (Frazer and Hensler, 1999). The axons of these neurons reach out to the central nervous system forming a neurotransmitter system called the serotonergic system. The first evidence of serotonergic involvement in OCD was found in the 1960s when clomipramine, a tricyclic antidepressant with serotonin reuptake blocking properties showed anti-obsessional properties (Fernandez and Lopez-Ibor, 1967). Subsequently, many other studies (Goodman et al., 1989; Chouinard, 1990; Jenike et al., 1990; Greist et al., 1995; Mundo et al., 2001) corroborated this claim. These studies led to exhaustive examination of the serotonin system and its function in OCD patients. Studies on 5-HT levels in whole blood (Thoren et al., 1980; Zohar et al., 1987) and platelets (Hanna et al., 1991; Hanna et al., 1995) of OCD patients though not definitive,

indicated serotonergic dysfunction. More evidence has come from pharmacological studies in which OCD patients were assessed after treatment with drugs like mCPP (Goodman et al., 1995), fenfluramine (Hollander et al., 1988), buspirone (Norman et al., 1994), sumatripan (Zohar et al., 1996) that stimulate 5-HT transmission. These studies have however also been inconclusive since many patients experienced worsening of symptoms. This could be due to basal hyperactivity of the serotonergic neurotransmission as a result of either the hypoactivity of the presynaptic, or hyperactivity of the post synaptic 5-HT receptors (Blier and Montigny, 1999). The efficacy of the chronic SSRI treatment regimen in many OCD patients could be due to the desensitization of the 5-HT autoreceptors (Blier and Montigny, 1999). Despite the consistent effectiveness of SSRIs in treating a significant subgroup of clinical responders, no agreement could be reached on the theory of serotonergic dysfunction as the primary cause of OCD. Several studies have refuted the serotonergic abnormality as a probable mechanism of the disorder (Berney et al., 2011; Maron et al., 2012). Moreover, 40-60% of the patients do not respond to SSRIs reflecting heterogeneity in clinical response to these drugs. Hence, the serotonin hypothesis might hold true for a subgroup of patients but not universally for all OCD patients.

ii) Dopamine hypothesis: Dopamine (DA) is derived from the amino acid phenylalanine and is the simplest neurotransmitter in the catecholamine family (Beaulieu and Gainetdinov, 2011). Dopamine projections reach to various regions of the brain that control executive functions, reward, motivation, reinforcement, motor control, etc. (Dayan and Balleine, 2002; Pierce and Kumaresan, 2006). Most important dopaminergic cell bodies with respect to OCD are those located in the ventral midbrain and consists of cell bodies A8 through A10. The nigrostriatal pathway consists of A9 cell bodies from substantia nigra pars compacta (SNc) to dorsal striatum and control voluntary movements (Hegarty et al., 2013). The A8 cell bodies project from ventral tegmental area (VTA) to nucleus accumbens in ventral striatum, while A10

develop from retrobural field (RRF) to the prefrontal cortex (Tzschentke and Schmidt, 2000). Both A8 and A10 cell bodies from the mesocorticolimbic system which play an important role in emotion and reward (Tzschentke and Schmidt, 2000). Considerable evidence exists about certain subtypes of OCD being mediated by dopaminergic systems (Pauls et al., 1995). Tourette's syndrome, a subtype of OCRD, is predominantly caused by dopaminergic abnormalities. This can be evidenced by the clinical response of patients to haloperidol and other dopamine antagonists (Shapiro et al., 1989). OCD patients with comorbid tic or Tourette's syndrome respond poorly to first line therapies and get more relief from adjuvant treatment with dopamine or DA/5-HT blockers (Ravizza et al., 1995; Stein et al., 1997a). Further, results from other studies suggest the importance of dopamine in fronto-striatal circuitry (Klanker et al., 2013), which is implicated in OCD. Deficiency in cognitive flexibility which includes reversal learning, task switching and attentional set shifting is often seen in OCD patients and is controlled by dopamine signaling (Chamberlain et al., 2006; Pauls et al., 2014). Dopamine depletion in brain regions like the caudate nucleus and orbitofrontal cortex leads to impairment in reversal learning and impaired extinction, respectively (Walker et al., 2009; Clarke et al., 2011). Animal studies have also substantiated this claim especially in rodents that exhibit excessive self-grooming and anxiety phenotypes (Campbell et al., 1999a; Berridge et al., 2005).

iii) Glutamate abnormality: The glutamate link in OCD was first proposed by Maria Carlsson (Carlsson, 2000, 2001). This was based on theoretical reasoning that considered OCD symptoms, functions of the CSTC circuitry and glutamate physiology. This theory has garnered further support from various studies that involve modulation of the glutamatergic system to look for symptom alleviation (Feusner et al., 2009; Haghighi et al., 2013; Rodriguez et al., 2013; Poyurovsky et al., 2010; Hussain et al., 2015; Pittenger, 2015). In rodents, knocking out genes like DLGAP-3 (Disks large-associated protein 3) (Welch et al., 2007) and SLITRK5 (SlitrK proteins) (Shmelkov et al., 2010) have shown abnormality in glutamatergic transmission leading

to expression of repetitive phenotypes, such as excessive grooming concomitant with anxiety. Treatment of these knockout mice with SSRIs has shown attenuation of these excessive behaviors indicating a possible relationship between serotonin and glutamate functionality (Welch et al., 2007; Shmelkov et al., 2010; Pauls et al., 2014). Excessive self-grooming has also been observed in glutamate transporter SLC1A1 knockout mice (Aoyama et al., 2006). Although these glutamatergic gene knockout studies suggest a role in OCD-like phenotypes, whether normal genetic variability in glutamatergic system plays a direct role remains to be elucidated.

iv) Peptide and steroid involvement: Several studies have pointed towards the involvement of hypothalamic peptides, such as oxytocin, vasopression and somatostatin in the pathophysiology of OCD (Salzberg and Swedo, 1992; Leckman et al., 1994b). However, further replications of these studies are required to understand their specific roles in the disorder because of contradictory evidence. One case study reported alleviation of OCD symptoms in patients treated with intranasal oxytocin (Ansseau, 1987), while other studies showed no effect (Epperson et al., 1996; den Boer and Westernberg, 1992). In addition, elevated levels of oxytocin have been seen in cerebrospinal fluid of OCD patients (Leckman et al., 1994a), while decreased activity has been hypothesized in autistic patients (Green et al., 2001). More animal studies are needed to better understand the role of these peptides in modulating neuronal circuitry that drive behavioral expressions seen in OCD.

Earlier onset in males when compared to females (Bogetto et al., 1999), bimodal distribution of onset in females and exacerbation of symptoms during various reproductive phases (Brandes et al., 2004) point towards the role of steroid hormones in OCD. Sex hormones are mainly steroids that are produced by the gonads (Brook, 1999). The most relevant are female sex hormones estrogen and progesterone, and the male sex hormone testosterone. These hormones regulate and influence global sexual functions like pregnancy, pubertal changes, sexual behavior, etc (Barth et al., 2015). Estrogen and progesterone have

neuromodulation effects in the central nervous system either through intracellular action or via neurotransmitter systems (Dreher et al., 2007; Karakaya et al., 2007; Benmansour et al., 2009). These sex steroids further metabolize into neurosteroids, which have anti-convulsive (Kokate et al., 1994), anti-depressant (Khisti et al., 2000) and anti-anxiety (Barbaccia, 2004) properties through modulation of GABA and glutamate receptors (Compagnone and Mellon, 2000).

The emphasis of sex steroids in influencing onset and exacerbation of OCD symptoms are more relevant to female OCD patients. This is mainly because women are at risk in developing the disorder during various reproductive phases (Alcafache, 2016). Very few studies have been conducted which provide contradictory evidence (Vulink et al., 2006). One study has shown more prevalence of OCD during menopause (Uguz et al., 2010), while another has demonstrated that the symptoms are more related to menarche and decrease during menopause (Guglielmi et al., 2014). Further, no literature evidence exists for acute conditions like surgical menopause, which is more abrupt and can mimic natural menopause (Rodriguez-Landa et al., 2015). Animal studies on the role of sex steroids in compulsive behaviors have also been very limited. Anti-compulsive effects of estradiol, allopregnanolone and progesterone has been established in handful of studies (Flaisher-Grinberg et al., 2009; Hill et al., 2007; Fernandez-Guasti et al., 2006; Umathe et al., 2009).

v) Cholinergic involvement: There is evidence of cholinergic system being involved in OCD (Lucey et al., 1993; Yankelevitch-Yahav and Joel, 2013). OCD patients typically report less smoking behavior when compared to other neuropsychiatric disorders (Abramovitch et al., 2014). It has been hypothesized that nicotine further activates the hyperactive fronto-striatal circuit resulting in exacerbation of symptoms. This is mainly through glutamate release by nicotinic receptor activation (Abramovitch et al., 2015). This theory has however been challenged by other studies which have shown significant clinical improvements in OCD patients augmented with nicotine (Pasquini et al., 2005). Since cholinergic projections innervate the

orbitofrontal cortex (Mesulam et al., 1986), which is one of the implicated regions in OCD (Menzies et al., 2008; Beucke et al., 2013), further investigation is required to understand the modulatory role of cholinergic system in obsessive-compulsive behaviors.

vi) Autoimmune and neuro-inflammatory hypothesis: The autoimmune hypothesis of OCD was first proposed by Allen et al. (Allen et al., 1995). This hypothesis was based on the review of existing literature sources which resulted in a correlation between infections with group A B-hemolytic streptococcus and worsening of OCD symptoms in children. This was followed by the characterization of the pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections including OCD (Swedo and Sydenham, 1994; Swedo et al., 1998). More recent evidence has emerged in this field that corroborates the autoimmune mechanism due to infection (Singer et al., 2012; Swedo et al., 2015).

1.1.4 Current Treatment Strategies

1.1.4.1 Pharmacotherapy

i) Monotherapy: The most predominantly used first line treatment for clinical responders are the selective serotonin reuptake inhibitors (SSRIs) (Kellner, 2010). SSRIs are a class of drugs that are thought to increase the extracellular levels of serotonin in the synaptic cleft by limiting its reuptake through the serotonin transporter (SERT) (Blakely and Barker, 1995). However, the exact mechanism of these drugs still remains to be elucidated. The concept of serotonergic agents in treating OCD gained momentum with the efficacy study of clomipramine in 1960s (Fernandez and Lopez-Ibor, 1967; Reynghe de Voxrie, 1968). Clomipramine is a first-generation serotonin reuptake inhibitor (SRI). SRIs are less selective than the SSRIs and can bind to other transporters such as norepinephrine (Tatsumi et al., 1997). Several studies since then have established the clinical effectiveness of clomipramine (Song et al., 1993; Mitchell et al., 1997; Anderson et al., 2000) with the most effective dose being 100-300 mg/day

(Montgomery et al., 1980). The SSRIs were introduced in the 1980s with fluvoxamine as the first SSRI that was tested on patients with clinical success (Goodman et al., 1989). Subsequently, other SSRIs such as fluoxetine (Montgomery et al., 1993; Tollefson et al., 1994), sertaline (Chouinard et al., 1990; Jenike et al., 1990), paroxetine (Weadon et al., 1993; Zohar and Judge, 1996), citalopram (Pallanti et al., 2002) and escitalopram (Stein et al., 2007) have also been introduced to treat OCD and have shown effectiveness in various clinical trials. Most of these drugs are used at doses of 50-200 mg/day (Chouinard, 1992; Fichter et al., 1996; Hollander et al., 2003; Irons, 2005).

In addition to SSRIs, 5-HT-noradrenaline reuptake inhibitors (SNRI) such as Venlafaxine have shown promise in clinical studies (Marazziti et al., 2003). Mirtazapine, a tricyclic antidepressant which acts on both the noradrenergic and serotonergic systems was found to be effective in some studies (Koran et al., 2005). Encouraging data has also emerged from phenelzine (monoamine oxidase inhibitor) (Vallejo et al., 1992), inositol (secondary messenger modulator) (Fux et al., 1996), cycloserine (glutamatergic agonist) (Storch et al., 2007) and morphine (Koran et al., 2005).

ii) Augmenting strategies and combinational treatments: Despite the widespread usage of SSRIs in treating OCD, 40-60% of the patients are refractory to the treatment (McDougle et al., 1993). Complete remission is not achieved and recurrence of symptoms is common (McDougle et al., 1993). Moreover, latent response times of 8-12 weeks for SSRIs have been a point of concern. Alternative therapeutic strategies like co-administration and augmentation have been used for these patients. Combination of SSRIs such as clomipramine with fluoxetine (Simeon et al., 1990; Browne et al., 1993) and clomipramine with citalopram (Marazziti et al., 2008) has been effective in some cases. Drug resistant patients have shown better response with paroxetine and pindolol, a beta blocker (Dannon et al., 2000). Augmentation with antipsychotics

like haloperidol and risperidone, which targets dopaminergic receptors, has been useful for some patients (McDougle et al., 1994; McDougle et al., 2000).

1.1.4.2 Non-Pharmacological Therapies

i) Psychotherapy: Exposure and response prevention (ERP) is a very popular non-pharmacological treatment option for OCD patients (Pittenger et al., 2005; Marazziti et al., 2012). Patients undergoing ERP typically are exposed to conditions that inflict obsessive thoughts but they are not allowed to engage in compulsive rituals (Meyer, 1966; Baer and Minichiello, 1998). This forced abstinence through repeated trials ultimately results in attenuation of engagement in compulsive behaviors. The exposure methods are either in-vivo or imaginary (Marazziti et al., 2012). Some of the examples are systemic desensitization, satiation/habituation, etc. (Marazziti et al., 2012). Response techniques to exposure stimulus include aversive methods or physical obstruction to engagement in overindulgent behaviors (Marazziti et al., 2012). This method has yielded results in 70-80% of the patients in clinical trials (Perse, 1988). Cognitive therapy has also been used to prevent intrusive thoughts and repetitive behaviors. Though based on the premise of ERP, cognitive therapy involves exposure and response preventions in the form of behavioral assessments (Freeston et al., 1997). The cognitive therapy component aids in improving engagement with reality, while disengaging from obsessive thoughts and thereby compulsive behaviors. However, a recent study has shown that 46% of the patients refused to undergo CBT therapy (Santana et al., 2013). In addition, both ERP and CBT are highly demanding and are used for severe cases patients with frequent relapses (Feusner et al., 2015).

ii) Deep brain stimulation: Deep brain stimulation is an invasive method which involves positioning of electrodes in target brain regions followed by electric stimulation (Gabriels et al., 2003). DBS is proposed for those patients who have stopped responding to pharmacological and behavioral interventions (Sedrak et al., 2013). The therapeutic effect of DBS is attributed to

its effectiveness in modulating abnormal activities and synaptic connectivity in the orbitofrontal cortex, anterior cingulate cortex, and striatum (Lujan et al., 2008). The response to this treatment strategy largely varies based on the target brain region for stimulation (Lujan et al., 2008).

iii) Neurosurgical methods: Most commonly used neurosurgical methods are bilateral anterior capsulotomy (Oliver et al., 2003), cingulectomy (Dougherty et al., 2002), lobotomy (Kim et al., 2003) and subcaudate tractotomy (Montoya et al., 2002). These ablative surgical techniques are predominantly used for very severe cases of OCD that remain resistant to all other treatment options (Yaryura-Tobias et al., 2000). Most of these surgeries are aimed at CSTC circuit components for remission of symptoms (Marazziti et al., 2012).

1.1.5 Animal Models of OCD

Several animal models have been developed in the past three decades that can provide insight into the neurochemical and neuroanatomical substrates of OCD (Alonso et al., 2015). Though it is impossible to recapitulate obsessions in animals due to the unique human topics that encompass the obsessional thoughts, animal models can provide crucial understanding of the phenomenology, such as compulsivity and stereotypical dimensions of the disease (Maio et al., 2014). To be considered a successful animal model of a human disorder, there should be analogous symptomology between the model and the human disease (face validity) and the treatment modalities should effectively reverse the symptoms (predictive validity). In addition, there should be similar molecular and physiological mechanisms (construct validity) between the animal models and the human disease it represents (McKiney and Bunney, 1969; McKinney, 1988). In the field of OCD, a successful animal model exhibits face validity that are typically repetitive, excessive and inappropriate behaviors. The predictive validity involves alleviation of these abnormal behaviors by treatment with SRIs and SSRIs, which are first line treatment options in human patients (Albelda and Joel, 2012). Since the exact neural

mechanisms of OCD are yet to be elucidated, construct validity of a model involves abnormality in CSTC circuitry, ovarian hormones, or the implicated neurotransmitter systems that can explain the behavioral correlates (face validity) and treatment response (predictive validity) (Albelda and Joel, 2012). Some of the current animal models used for studying OCD are discussed as follows:

1.1.5.1 Genetic Models

i) *5-HT2c receptor knockout mouse model:* The serotonin receptor type 2c (5-HT2c) receptor knockout mouse was generated by Chou-Green et al. (2003). Knocking out the 5-HT2c receptor resulted in compulsive-like chewing of nonnutritive clay, patterned chewing of plastic mesh screen and reduced habituation or perseveration to the head-dipping task (Chou-Green et al., 2003). This organized chewing behavior represents compulsive checking, ordering, smoothing or washing behaviors in humans. The head-dipping is considered analogous to compulsive checking (Chou-Green et al., 2003). A recent study with this mouse model revealed that they increased reversal learning by reducing influence on non-reward associations (Nilsson et al., 2013). Despite showing promise in understanding the role of 5-HT2c receptor role in driving behavioral outcomes, the model lacks predictive validity.

ii) Hoxb8 mutant mice: The Hoxb8 gene is a member of the mammalian homeo box containing complex transcription factor responsible for neural development (Greer and Capecchi, 2002). Greer and Capecchi (Greer and Capecchi, 2002) developed the Hoxb8 mutant mice which exhibits excessive grooming behavior. This excessive grooming phenotype can be compared to trichotillomania and certain OCD subtypes (Frank et al., 2002). Moreover, Hoxb8 is predominantly expressed in brain regions implicated in OCD making it a good candidate for understanding molecular underpinnings behind abnormal stereotypic behaviors. Unfortunately, the predictive validity of this model has not been established (Wang et al., 2009).

iii) *D1CT-7 mutant mice*: DICT-7 mice express cholera toxin A1 subunit within a subset of dopamine D1-receptor expressing neurons in the cortical-limbic region (Burton et al., 1991; Smicun et al., 1999). DICT-7 mice exhibit a constellation of compulsive behaviors such as, repetitive episodes of normal behaviors, excessive grooming and biting of siblings and multiple episodes of leaping (Smicun et al., 1999). These behaviors provide face validity for compulsive-like Tourette syndrome (Nordstrom and Burton, 2002). However, the DICT-7 mice also exhibit limbic seizures that are not linked to OCD behaviors (Campbell et al., 2000) indicating overlapping phenotypes. The predictive validity of this model is also yet to be determined.

iv) Dopamine transporter knockout mice: The dopamine transporter (DAT) knockout mouse is a mutant mouse in which the pre-synaptic DAT has been knocked down (Berridge et al., 2005). This downregulation of DAT expression results in elevated levels of pre-synaptic dopamine (extracellular) which leads to hyperactive and persevarative behaviors such as rigid grooming chain patterns (Zhuang et al., 2001; Berridge et al., 2005). This rigid syntactic grooming behavior is thought to resemble sequential super-stereotypy in OCD and Tourette syndrome (Sheppard et al., 1999). The DAT knockout mice however also exhibit behavioral abnormalities that are related to other disorders, such as ADHD and mania (Joel, 2006).

v) Aromatase knockout (ArKO) mice: The aromatase knockout mice were originally developed to understand the influence of estradiol in sexual differentiation (Fisher et al., 1998). Knocking out aromatase activity causes inhibition of estradiol synthesis from testosterone. This specific knockdown of aromatase activity results in excessive wheel running and grooming behavior in males but not females and estrogen administration results in attenuation of these phenotypes (Hill et al., 2007). The onset of the stereotypical behaviors in ArKO mice is seen only at 6 months of age which is complemented by a decline in catechol-o-methytransferase (COMT) protein expression in the hypothalamus (Wang et al., 2009). Though reduced function of COMT in male OCD patients has been seen in a few studies (Pooley et al., 2007; Schindler

and Anghelescu, 2007), there are some other potential problems with this mouse model. The lack of a compulsive-like phenotype in females and the late onset (6 months), which correspond to the early to late adulthood years in humans, questions the predictive validity of the model (Wang et al., 2009). Moreover, it is not yet determined if the reductions in COMT levels drive the compulsive-like behaviors in the mice. Another potential drawback is the lack of chronic treatment studies with first line treatments.

vi) Sapap3-mutant mice: SAP90/PSD95-associated protein-3 (SAPAP-3) is a scaffolding protein that is expressed in the postsynaptic membrane of the striatal region. Knocking out SAPAP-3 in mice resulted in exaggerated grooming behavior along with anxiety-like behaviors in open field and elevated plus maze tests (Welch et al., 2007). The predictive validity of this model has also been established through subchronic administration of fluoxetine which attenuates the grooming and anxiety-like behaviors. However, the predictive validity of this model has been determined through a fluoxetine dose of 5 mg/kg/day for only 6 days. This is a substantially low dose when compared to the recommended dose used for humans (Wang et al., 2009). It is also noteworthy that in other animal models higher doses (10 to 20 mg/kg/day) are required for at least 12-14 days for attenuation of anxiety and depression-like behaviors (Dulawa et al., 2004; Yalcin et al., 2008). Also, the role of SAPAP-3 protein in influencing OCD in humans is yet to be determined (Wang et al., 2009) and therefore the construct validity cannot be substantiated.

vii) *Slitrk5 knockout mice*: The Slitrk5 gene belongs to a family of integral membrane proteins, which regulate neurite outgrowth during developmental phases (Aruga and Mikoshiba, 2003). Slitrk5 knockout mice exhibit increased neuronal activity in the orbitofrontal cortex and striatum. As a result of this abnormal neural activity the mice develop excessive self-grooming, increased marble burying and anxiety-like behaviors (Shmelkov et al., 2010). The predictive

validity of this model has also been verified through repeated fluoxetine treatment, which attenuates the compulsive phenotype (Shmelkov et al., 2010).

1.1.5.2 Drug Induced Models

Drug induced models of OCD are based on behavioral alterations due to pharmacological manipulations of the neurotransmitter systems that are thought to be implicated in OCD. Two of the most widely studied drug induced models are:

i) 8-OH-DPAT-induced spontaneous alternation: The concept of drug induced attenuation of normal, sequential and successive exploration of novel places by rats was first proposed by Yadin et al. (Yaldin et al., 1991). Based on this concept, rodent models have been established that involve acute administration of 8-OH-DPAT, an agonist of the 5-HT1a receptor. This treatment with 8-OHDPAT decreases spontaneous alternation in mice and rats (Seibell et al., 2003; Arora et al., 2013) modeling indecision in humans, which is a specific aspect of OCD (Joel, 2006). The 8-OH-DPAT induced reduction in spontaneous alternation is restored by repeated fluoxetine or clomipramine treatment but not by tricyclic antidepressant, desipramine (Yadin, 1991; Fernandez-guasti et al., 2003) and can also be influenced by ovarian hormones (Fernandez-guasti et al., 2003). However, spontaneous alternation is observed in many other psychiatric and neurological manifestations such as Parkinson's disease and schizophrenia (Albelda and Joel, 2012). Hence, a conclusion cannot be reached as to whether the face validity of the model is exclusive to OCD.

ii) *Quinpirole-induced compulsive checking*: This model was produced by chronic treatment (5 weeks) of rats with quinpirole (0.5 mg/kg), a D2/D3 agonist (Szechtman et al., 1998). Following treatment, the rats are allowed to explore an open field with four small objects placed at a fixed location in the field. Quinpirole treated rats typically display two locales in which they stop more frequently than others and perform ritualistic motor acts when they stop at

these objects (Ben-Pazi et al., 2001; Szechtman et al., 1998, 2001). This persistence behavior that is dedicated to specific objects and ritual-like motor activity is considered analogous to compulsive checking in human patients (Szechtman et al., 2001). This model has also shown response to clomipramine treatment which reduces the checking behavior partially or momentarily (Szechtman et al., 1998).

1.1.5.3 Spontaneous Models

Spontaneous models use the concept of innately occurring stereotypic behaviors, motor behaviors or adjunctive behaviors that are induced through behavioral manipulations (Woods et al., 1993; Altemus et al., 1996; Alonso et al., 2015). Some of the most widely studied spontaneous models are:

i) Marble burying behavior: The marble burying behavior is one of the most widely studied models to assess compulsive-like phenotypes. The concept of marble burying utilizes the natural tendency of mice or rats to bury harmless or noxious objects in the bedding provided to them (Joel, 2006). Since studies (Njung'e and Handley, 1991; Gyertyán, 1995; Londei et al., 1998) have shown that rodents typically do not tend to avoid the marbles, marble burying is a good indicator of a compulsive-like but not an anxiety-like phenotype (Gyertyán, 1995; Londei et al., 1998). The compulsive theory of marble burying is based on the fact that non-reactivity of the marbles fails to provide stimuli for ending the investigation leading to frustrated compulsive burying (Szechtman and Woody, 2006; Joel, 2006). Marble burying can be mapped to the inability to realize the task completion in OCD patients (Szechtman and Woody, 2006), thereby establishing its face validity. Several studies have also shown predictive validity of this model through SSRI treatment regimens (Takeuchi et al., 2002; Greene-Schloesser et al., 2011). The construct validity of this model is moreover supported through studies where ovarian hormones, neurosteroids and the estrous cycle have been found to influence the burying behavior (Schneider and Popik, 2007; Llaneza and Frye, 2009; Umathe et al., 2009).

ii) Signal attenuation model: The signal attenuation model is based on the concept of deficiency in the feedback of normal goal directed behavior (Gray, 1982; Baxter, 1999; Szechtman and Woody, 2006). Using this concept, rats are trained to exhibit compulsive lever pressing. This is achieved by trainings rats to lever press for food in the presence of a paired stimulus. The stimulus acts as a feedback cue for the rats to press the lever. The rats are then presented with an attenuation phase during which repeated stimuli are provided without food. Finally, rats are tested for the number of lever press in the extinction phase by presenting the stimulus without food (Joel and Avisar, 2001; Joel et al., 2001; Joel and Doljansky, 2003). The predictive validity of the model has been assessed using acute doses of paroxetine and fluvoxamine which reduced the lever pressing behavior. The tricyclic antidepressants however have no effect on the number of lever presses (Joel et al., 2004). The construct validity of the model has also been investigated in studies where ovarian hormones have been found to modulate lever-pressing (Flaisher-Grinberg et al., 2009). One of the major shortcomings of this model is the lack of predictive validity for chronic administration of first line treatments (Alonso et al., 2015).

iii) *Schedule-induced polydipsia model*: In this paradigm, rats are food deprived and trained to collect food only after a fixed time interval while having free access to water. Rats eventually exhibit polydipsic behavior whereby they consume five to ten times more water than control rats not exposed to the training (Woods et al., 1993). This excessive water intake is repetitive and can be considered a compulsive-like phenotype (Alonso et al., 2015). Chronic administration of SSRIs has also shown to reduce this polydipsia behavior establishing the predictive validity of this model (Woods et al., 1993). Neurobiologically, activating 5HT2c receptors with agonists reduces the polydipsic behavior indicating serotonergic involvement and possible construct validity of the model (Rosenzweig-Lipson et al., 2007).

iv) Spontaneous stereotypic deer mice model: The deer mice exhibit a unique set of stereotypic behaviors such as vertical jumping, backward somersaulting and patterned running (Powell et al., 1999). Depending on the frequency of occurrence of these behaviors, deer mice are categorized into high, low and non-stereotypic types. These behaviors have been used in studies to understand compulsive phenotypes (Wolmarans et al., 2016; Bechard et al., 2017). Though chronic administration of fluoxetine decreases these stereotypic behaviors (Korff et al., 2008), there are some drawbacks with this model. The studies with deer mice have used both males and females but the influence of sex in these studies has not been discussed. Also, a study with mCPP and quinpirole in these mice has caused attenuation of the stereotypic behaviors (Korff et al., 2008). This is puzzling since both mCPP and quinpirole are known to exacerbate compulsive-like behaviors in rodents (Erzegovesi et al., 2001; Tsaltas et al., 2005; Alonso et al., 2015). Moreover, the predictability of this model is questionable since it does not exhibit uniform compulsive-like phenotypes. Only a certain percentage of the offsprings exhibit excessive nest-building (Wolmarans et al., 2016b), while marble burying is not an indicator of compulsive-like phenotypes in these mice (Wolmarans et al., 2016).

v) Bidirectional selection for nest building behavior: This mouse model was developed through bidirectional selection for high and low levels of nest-building from an HS/ibg outbred mouse stock population (Lynch, 1980; Bult and Lynch, 2000). The HS/ibg strain was derived through crossing of eight house mouse (*Mus musculus)* inbred strains (A, AKR, BLB/c, C3H/2, C57BL, DBA/2, Is/Bi, RIII; McClearn et al 1970; Lynch, 1980). Bidirectional selection resulted in three levels of nest-building behavior (with two replicate strains within each level). The replicates within each level of nest building were maintained as separate strains, i.e., not interbred with the other replicate strains, but subjected to the same selection regime (Lynch, 1980; Bult and Lynch, 2000). This resulted in two BIG (BIG1 and BIG2) strains that consistently display high and excessive levels of nesting with a forty-fold difference in the amount of cotton

used when compared to the two SMALL strains which display very low levels of nesting. The SMALL strains are therefore considered as non-compulsive. The two randomly-bred strains serve as a selection Control and show intermediate levels of nesting (Bult and Lynch, 2000). The BIG strains also exhibit compulsive-like marble burying behavior (Greene Schloesser et al., 2011). A prior study has indicated that the BIG1 male strains have face and predictive validity for compulsive-like phenotype to study OCD in humans (Greene Schloesser et al., 2011). This was established by comparing behavioral responses of the BIG1 strains treated separately with fluoxetine, clomipramine and desipramine. There was attenuation of compulsive-like nesting and marble burying behavior in the BIG1 males treated with chronic fluoxetine and clomipramine. However, desipramine showed no significant differences (Greene Schloesser et al., 2011). The current thesis is a continuation of this work that investigates the role of strain and sex differences in compulsive-like condition. The research aims at probing into the role of ovarian sex steroids and postpartum phases in compulsive-, affective- and cognitive- behaviors. The role of cholinergic system in modulating compulsive-like behaviors is also elucidated through this thesis work.

1.2 Research Objectives

Sex differences and genetic variations are one of the main contributing factors to the complex heterogeneity associated with OCD (Arnold et al., 2006; Labad et al., 2008; Nestadt et al., 2010; de Mathis et al., 2011; Grunblatt et al., 2014). Currently, there is a lack of studies that emphasize the influence of sex differences on compulsive-like behaviors in model organisms. Most of the model systems of OCD discussed above lack sex based comparisons and do not consider the role of ovarian steroids in influencing behavioral outcomes. Understanding of onset and exacerbation of OCD in females during periods of physiological challenges, such as pregnancy, postpartum and menopause, is also very limited (Arnold, 1999; Vulink et al., 2006; Guglielmi et al., 2014; Challacombe et al., 2017). Most of these cohort based studies also do

not report OCD associated comorbidities and cognitive impairments. This is further compounded by genetic variations among patient populations which might result in OCD subsets with specific obsessive-compulsive symptoms and co-morbid behaviors (den Braber et al., 2016). This heterogeneity also results in drug resistance (Erzegovesi et al., 2001) and the current first line therapies available are not effective against clinical non-responders. Most of the existing rodent models do not represent the complex heterogeneity associated with OCD. Deciphering the phenotypic expressions of compulsions and complementary neurobiological mechanisms between compulsive-like mouse strains of opposite sex will be essential for better understanding of complex factors that contribute to this heterogeneity. The current research therefore aimed at investigating additional face, predictive and construct validity of the spontaneous mouse model of OCD first described by Greene-Schloesser et al (Greene-Schloesser et al., 2011) with the following aims:

1.2.1 Aim I: Behavioral characterization of a spontaneous compulsive-like mouse model based on strain and sex differences to understand clinical heterogeneity associated with OCD. This research aimed at establishing the face validity of the mouse model (Greene-Schloesser et al., 2011) to study OCD heterogeneity.

There is compelling genetic basis of OCD from clinical studies (Abelson et al., 2005; Shugart et al., 2006; Samuels et al., 2007; Hanna et al., 2007; Ross et al., 2011; Mathews et al., 2012; Goodman et al., 1990; Taylor., 2013; Stewart et al., 2013). In addition, the importance of sex differences in influencing onset, symptomology, severity and drug response has also been established (Noshirvani et al., 1991; Bogetto et al., 1999; Kalra and Swedo, 2009). However, there is lack of understanding whether there is a combined effect of genetic variations and sex differences that interact with each other to influence OCD. There is also a lack of understanding on how genetic variations and sex differences attribute to the complex heterogeneity of OCD including the expression of compulsive and comorbid behaviors and drug response. Previously,

only one study has been conducted on female mouse strains (C57BL/6J) that were treated with quinpirole to induce compulsive-like behaviors (de Haas et al., 2012). The study indicated that genetic background is essential in expression of compulsive-like behaviors and strain comparisons can provide a better understanding of various forms of compulsivity. However, the study used a wildtype strain and looked at the effect of strain differences only in females, but not males (de Haas et al., 2012). It also did not account for the associated affective (anxiety-like and depression-like) and cognitive (memory function) phenotypes. Considering that OCD is a complex heterogeneous disorder which varies based on sex differences and genetic background of patient populations I hypothesized that:

Hypothesis: There will be interplay of strain and sex differences in influencing compulsive-, anxiety-, cognitive- and depression-like behaviors among male and female compulsive-like mouse strains. This complex interplay of strain and sex will provide a better understanding of the heterogeneity of OCD associated with symptomology and drug response enhancing the face and predictive validity of this mouse model.

1.2.2 Aim II: Strain and trait specific attenuation of compulsive-like behavior by fluvoxamine in a non-induced mouse model (Greene-Schloesser et al., 2011) of obsessive-compulsive disorder. Through this aim I wanted to investigate behavioral responses of two different compulsive-like mouse strains in response to a first line treatment, fluvoxamine for OCD.

Around 40-60% of OCD patients do not respond to first line treatments (Ravizza et al., 1995). Even patients who are categorized as clinical responders continue exhibiting residual obsessions and compulsions (Lack, 2012). Through animal studies, a better understanding of the efficacy of first line treatments on specific compulsive traits is required. This can provide answers to such high drug resistant rates among patients. Investigating drug intervention in

mouse strains exhibiting spontaneous compulsive-like behaviors can be a valuable starting point for determining if there is any role of genetic background and compulsive traits in influencing drug response. Hence, I tested the hypothesis that:

Hypothesis: There will be a differential response to compulsive-like and anxiety-like behaviors in compulsive-like mouse (Greene-Schloesser et al., 2011) strains treated with increasing doses of SSRI, fluvoxamine.

1.2.3 Aim III: This aim involved investigating how ovarian sex hormones influence compulsive, affective and cognitive functions in a spontaneous mouse model of OCD (Greene-Schloesser et al., 2011). Through this aim, I intended to establish the construct validity of the mouse model on the premise of sex hormonal regulation of neurobiological mechanisms implicated in OCD.

There is currently no understanding as to how acute menopause due to surgical removal of ovaries influences compulsive, affective and cognitive behaviors in normal versus OCD patients. Moreover, the role of hormone therapy and response to hormone therapy due to genetic variations among OCD patient populations is not explored. Clinical and genetic data from human and animal studies corroborate the concept of sexual dimorphism in OCD (Karayiorgou et al., 1997; Camarena et al., 2001; Enoch et al., 2001; Lochner et al., 2004; Denys et al., 2006; Dickel et al., 2007; Hill et al., 2007; Labad et al., 2008; Flaisher-Grinberg et al., 2009; Torresan et al., 2009). There is prevalence of certain OCD types among females, such as cleaning compulsions, when compared to males (Noshirvani et al., 1991; Bogetto et al., 1999) and the drug response also varies (Mundo et al., 1999). Females typically experience a late onset when compared to males and display a bi-modal distribution with peaks occurring at ages that represents puberty and child bearing years (Brandes et al., 2004). These are physiological stages during which there are alterations in female sex hormonal levels primarily

produced by the ovaries. Few research studies have contradictory evidence on onset and exacerbation of OCD among females during various physiologically challenging phases like postpartum, menarche and menopause (Uguz et al., 2010; Guglielmi et al., 2014). Further, a limited number of animal studies has been undertaken to understand the role of ovarian steroids in compulsive-like behaviors (Fernández-Guasti et al., 2006; Hill et al., 2007; Flaisher-Grinberg et al., 2009). One study has shown that administration of estradiol to pre-pubertal rats is anti-compulsive (Flaisher-Grinberg et al., 2009), while another study showed that male mice with estrogen deficiency results in compulsive-like grooming (Hill et al., 2007). A study on rats has shown a combined effect of estradiol and progesterone in attenuating compulsive behavior (Fernández-Guasti et al., 2006). These studies do not reflect acute ovarian steroid deprivation, associated anxiety-like and cognitive-like behaviors in compulsive-like condition, and also do not explore the role of strain differences on steroidal influence of these behaviors. Hence using the spontaneous mouse model of OCD (Greene-Schloesser et al., 2011), I proposed that:

Hypothesis: Surgical menopause through acute ovariectomy will exacerbate compulsive-like, anxiety-like and cognitive-like behaviors in the compulsive-like mouse strains. I also hypothesized that the ovarian sex hormones estrogen and progesterone will modulate compulsive-like, anxiety-like and cognitive-like behaviors and this modulation will vary based on strain differences.

1.2.4 Aim IV: Elucidating the neurobiological mechanisms of compulsive-like, anxiety-like and depression-like behaviors during post-partum lactation in the spontaneous mouse model of OCD (Greene-Schloesser et al., 2011).

The physiology of OCD during post-partum in female patients is poorly understood. This is of importance considering that sex differences attribute to the phenotypic expression, heterogeneity and drug response variations in OCD (Noshirvani et al., 1991; Bogetto et al.,

1999; Mundo et al., 1999). Moreover, anxiety and depression, which are prevalent co-morbid conditions in OCD (Crino and Andrews, 1996; Goodwin, 2015), are known to precipitate during the post-partum phase in females (Smith et al., 2011; Alipour et al., 2012). Serotonergic systems are thought to play a critical role in the expression of obsessions and compulsions (Zohar et al., 2012; Pauls et al., 2014). In a recent study, lactation in wild-type female C57BL/6 mice resulted in a decrease of serotonergic neurons in the dorsal raphe nucleus of the brain. However, the serum levels of serotonin were higher during lactation (Jury et al., 2015). This variation in brain versus serum serotonin correlated with better behavioral responsiveness to SSRI treatments in lactating females when compared to nulliparous and postpartum females (Jury et al., 2015). Further, dopamine cell bodies (A12) of arcuate nucleus projecting to pituitary (Bjorklund and Nobin, 1973) negatively regulate lactation, while oxytocin neurons from paraventricular region (PVN) of hypothalamus influence lactation positively (Zingg and Lefebvre, 1988). This dual regulation of lactation and its impact on behavioral outcomes is not well understood. Lactation mediated neurobiological mechanisms during psychiatric disorders such as OCD is also poorly understood. Hence, this aim investigated if postpartum lactation has anti-compulsive, anti-anxiety and anti-depression like effects when compared to non-lactating and nulliparous females in compulsive-like mice. Due to the dual regulation of lactation by both dopamine and oxytocin, this aim also investigated if lactation-mediated behavioral outcomes in compulsive-like mice are mediated by dopamine and/or oxytocin. Through these aims I tested the hypothesis that:

Hypothesis: Lactating compulsive-like mice will exhibit less compulsive-, anxiety- and depression-like behaviors and will have enhanced responsiveness to SSRI treatments when compared to their non-lactating postpartum counterparts and nulliparous controls. These behavioral responses in lactation phase will be differentially regulated by dopamine and/or oxytocin.

1.2.5 Aim V: This aim probed the therapeutic role of desformylflustrabromine (dFBr) for attenuation of compulsive-like behaviors through positive allosteric modulation of the α4β2 neuronal nicotinic receptor subtype.

Human and rodent studies have indicated the cholinergic involvement in OCD (Lucey et al., 1993; Yankelevitch-Yahav and Joel, 2013). However, the results have been contradictory. Some studies have shown that nicotinic activation of already hyperactivated fronto-striatal circuit results in exacerbation of OCD symptoms (Abramovitch et al., 2015). On the other hand, one study has established nicotine augmentation as a therapeutic option for OCD patients (Pasquini et al., 2005). There is lack of understanding on how specific nicotinic receptor subtypes influence phenotypic expressions in OCD patients and how their modulation might provide further knowledge on their role in influencing compulsive-like behaviors. Considering that α4β2 subtype of neuronal nicotinic receptor are prevalent in brain regions implicated in OCD, addiction and mood disorders (Wise, 2009; Maskos, 2010; Quik et al., 2013), I decided to investigate whether modulation of this subtype of receptor with desformylflustrabromine, a novel positive allosteric modulator will affect compulsive-like behaviors in the mouse model. Based on this premise, I hypothesize that:

Hypothesis: Positive allosteric modulation of α4β2 subtype of nicotinic receptors will modulate compulsive-like and anxiety-like behaviors in the spontaneous mouse model.

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Chapter 2: Strain and sex based characterization of behavioral expressions in non-induced compulsive-like mice.[1]

2.1 Abstract

There is currently a lack of understanding how genetic background and sex differences attribute to the heterogeneity of obsessive-compulsive disorder (OCD). An animal model of compulsive-like behaviors has been developed through bidirectional selection of house mice (*Mus musculus*) for high (big cotton nests; BIG mice) and low levels (small nests; SMALL mice) of nest-building behavior. The BIG male strains have predictive and face validity as a spontaneous animal model of OCD. Here, we evaluated compulsive-, anxiety-, cognitive-, and depression-like behaviors among male and proestrus female replicate strains each of BIG (BIG1, BIG2) and SMALL (SML1, SML2) nest-builders, and randomly-bred Controls (C1, C2). BIG1 and BIG2 males and females had higher nesting scores when compared to SMALL and Control strains. Male BIG1 and BIG2 strains showed more compulsive-like nesting than BIG1 and BIG2 proestrus females, which was not observed among the other strains. Nesting scores were also different between BIG replicate male strains. A similar pattern was observed in the compulsive-like marble burying behavior with BIG strains burying more marbles than SMALL and Control strains. Significant replicate and sex differences were also observed in marble burying among the BIG strains. The open field test revealed replicate effects while the BIG strains showed less anxiety-like behavior in the elevated plus maze test compared to the SMALL strains. For novel object recognition only the Control strains showed replicate and sex differences. In the depression-like forced swim test proestrus females demonstrated less depression-like behavior than males. BIG and SMALL nest-building strains had a higher corticosterone stress response than the Control strains.

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Together these results indicate a strong interplay of genetic background and sex in influencing expression of behaviors in our compulsive-like mouse model. These results are in congruence with the clinical heterogeneity of OCD.

2.2 Introduction

Obsessive-compulsive disorder (OCD) is a debilitating psychiatric condition characterized by invasive and persistent thoughts (obsessions) and repetitive behaviors (compulsions) [4]. OCD has an estimated lifetime prevalence of 2.3% in the United States [97]. The majority of patients suffering from OCD perform repetitive rituals to mitigate uncomfortable feelings of anxiety [32]. Others engage in repetitive behaviors due to subjective sensations also called sensory phenomena [79]. Obsessions can be associated with contamination, fear and symmetry, while compulsions include hand washing, checking or counting [88]. Such excessive ritualistic behaviors become distressing and significantly interfere with daily functioning [4]. OCD exhibits a large behavioral repertoire with high rates of repetition and provides an ethological basis for studying compulsive-like behaviors in animal models of OCD [11]. Though inappropriate for investigating the entire OCD spectrum because obsessions cannot readily be assessed, animal models can provide deep insight into various forms of compulsivity [74].

Sex differences in OCD [14,29,63] is thought to contribute to the heterogeneity of OCD [59]. Age of onset [14], phenomenology [59] and comorbidity [69] are some of the gender related differences that have been observed. Males usually have an earlier onset for most symptom dimensions of OCD than females [23,72,82,83]. Clinical studies have indicated that sexual, religious and symmetrical obsessions, and compulsive checking and ordering/arranging are more frequently seen in males than females, while females tend to exhibit more obsessions for contamination and compulsive cleaning than men [30,62,72,108].

In females, ovarian hormones may play a critical role in modulating obsessions and compulsions [113]. Fluctuations in the female hormonal cycle may contribute to increased risk of onset and exacerbation of OCD symptoms at certain reproductive events, including premenstruum phase, menstrual cycle, pregnancy, and postpartum [1,63,73,109,113]. Few rodent studies have established that the female sex hormone estrogen can modulate

compulsive-like behaviors [33,37,58]. The proestrus stage of the estrous cycle in female mice has higher circulating levels of estrogen influencing anxiety-, cognitive- and depression-like behaviors [106,116] and, therefore, the comparison between males and proestrus females for compulsive-like behaviors may provide additional ways to gain important insights using animal models of OCD.

Additionally, inbred female mouse strains have shown significant differences in expression of drug induced compulsive-like behavior [27], indicating that strain comparisons can be valuable for understanding expression of compulsivity. This is important considering the compelling genetic basis of OCD from various human studies [25,51,52,56,76,96,105], which coupled with the sex differences can provide crucial clues about the complex interactions of these two elements in expression of compulsive and affective behaviors.

According to Maio et al. [74], comparing our compulsive-like mouse strains is valuable for understanding the disorder. Compulsive-like behavior in our mice is defined as excessive and repetitive expression of otherwise normal behaviors, i.e., nest-building and marble burying. For example, nest-building in the compulsive-like strains (BIG1 and BIG2) involves rapid and repeated movements of the front legs and mouth to pull excessive amount of cotton through the cage top metal bars over extended periods of time [50], which shows face validity with repetitive behaviors in OCD [79]. This rapid, excessive and repetitive nesting behavior is not observed in the Control and SMALL strains. The BIG strains also exhibit predictive validity as a spontaneous non-induced model of OCD-like behaviors through attenuation of these compulsive-like behaviors with fluoxetine and clomipramine treatment, which are first line treatments for OCD [50]. In addition, the tricyclic antidepressant desipramine, which is not effective in treating OCD, did not change the compulsive-like behaviors in the compulsive-like mice [50]. These strains were developed by bidirectional selection for high and low levels of nest-building behavior [19,71] using a stock population, i.e., HS/Ibg outbred strain, that was derived from a cross

among eight inbred house mouse, Mus musculus, strains, i.e., A, AKR, BLB/c, C3H/2, C57BL, DBA/2, Is/Bi, and RIII [71,77].

Bidirectional selection resulted in three levels of nest-building behavior (with two replicate strains within each level). The replicates within each level of nest building were maintained as separate strains, i.e., not interbred with the other replicate, but subjected to the same selection regime. Using replicate strains is important to make sure that responses to artificial selection are due to selection and not the result of founder effects or random genetic drift when comparing the different levels of selection, i.e., selection for building big nests or small nests, or randomly bred [17,19]. The two BIG strains consistently display high levels of nesting with a forty-fold difference in the amount of cotton used when compared to the two SMALL strains which display very low levels of nesting [19,71]. The two randomly-bred strains serve as a selection Control and show intermediate levels of nesting [19,71].

Compulsive-like nesting in our mice has a genetic factor with about 30% of the variation in behavioral expression among individuals due to additive genetic factors and about 70% of the variation due to environmental factors [19,71]. This estimate of heritability of nest building falls within the range of heritability estimates of 0.36 [111], 0.37 [26], 0.23–0.58 [60], and similar estimates for different types of OCD [16]. In addition, quantitative genetic analyses have revealed nesting behavior to be a highly polygenic trait [19,71], which is consistent with OCD in humans likely being influenced by many different genes [57,89,101]. Considering the heterogeneity and sex factors in OCD and the behavioral and genetic characteristics of our mouse model of OCD, we aimed to investigate the effects of genetic background and sex on the compulsive-, anxiety-, cognitive- and depression-like behaviors for a better understanding of factors influencing compulsivity.

2.3 Methods

The University of Alaska Fairbanks Institutional Animal Care and Use Committee approved the animal care and experimental procedures (IACUC assurance number 497513).

2.3.1 Mice

2.3.1.1 Husbandry

Mice were housed in polypropylene cages (27 × 17 × 12 cm) with wood shavings in a temperature (22 ± 1 °C) and humidity (60–80%) controlled room of the Biological Research and Diagnostics (BiRD) Facility vivarium on a 12:12 h light-dark cycle. Pups were weaned at 19–21 days of age and housed with same-sex and same-strain littermates until the end of all experiments. Food (Purina Mills, Lab Diet Mouse Diet #5015, St. Louis, MO) and water were available ad libitum.

2.3.1.2 Mouse strains

Randomly-bred Control strains (C1, C2), BIG strains selected to build big nests (BIG1, BIG2) and SMALL strains selected to build small nests (SML1, SML2) [17,18,19] were used. The BIG strains express higher levels of compulsive-like behaviors, i.e., nest building and marble burying, when compared to the SMALL strains [50], while the Control strains are intermediate [19,71]. Male and female mice (Mus musculus) of these six different mouse strains were used for the behavioral experiments (C1 males n = 20; C1 females n = 21; C2 males n = 20; C2 females n = 17; BIG1 males n = 21; BIG1 females n = 22; BIG2 males n = 20; BIG2 females n = 14; SML1 males n = 19; SML1 females n = 21; SML2 males n = 19; SML2 females n = 20).

2.3.2. Estrous cycle

Female mice in proestrus were used for the study. The proestrus stage was determined daily by both visual and vaginal cytology methods [20]. All females from all the strains were

visually inspected daily. Vaginal cytology was performed on those females that showed visual signs of proestrus. The females for which proestrus was confirmed through cytology were subjected to behavioral testing the same day. This procedure was conducted daily until desired sample sizes were achieved for a specific test. When the females cycled to their next proestrus, which was typically on the 4th or the 5th day, they were subjected to the next behavioral test in the schedule and this cycle was continued until all behavioral tests were completed.

2.3.3 Experimental design

All mice were at least 60 days old at the start of the experiment. All behavioral tests were done in the light phase of the light-dark cycle. All males underwent behavioral tests once in every 5 days consisting of nest building (on day 1), marble burying (on day 6), open field (on day 11), elevated plus maze (on day 16), novel object recognition (on day 21) and forced swim test (on day 26). For females, behavioral tests were conducted every 4–5 days depending on their proestrus stage. Males and females were tested on this schedule until desired sample sizes were achieved. A gap of 4 days for males and 3–4 days for females between each behavioral test minimized the behaviors interfering with each other.

For nest-building, animals were housed individually in cages and, therefore, following data collection after 24 h, they were returned to home cages with their littermates and kept for 4 days to negate any isolation effects that could influence performance in subsequent tests [55]. For marble burying, open field, elevated plus maze, and novel object recognition the mice were singly housed just before testing. After testing they were returned to their home cage with their littermates. Upon reintroduction to their littermates, some fighting was observed within the first hour, which was similar to fighting observed after a normal cage change, which should not have interfered with the subsequent behavioral assessment considering a 3–4 day gap between tests. Immediately after the forced swim test, trunk blood was collected to determine stress-

induced plasma corticosterone levels. An observer blind to the conditions of experimental animals and the hypothesized outcome of the study collected all data.

2.3.4 Measuring compulsive-like behavior

2.3.4.1 Nest-building test

Nest-building behavior was used as a measure of the compulsive-like phenotype of the mice [50]. Male and female mice of all the strains were singly housed and provided with a pre-weighed cotton roll in the cage-top food hopper. After 24 h, the cotton roll was removed and weighed [17,18,19,71]. The total nesting score was defined as the amount of cotton used over the 24-h period. Generally, all the mice incorporated the cotton into their nest. The more cotton was used, the more elaborate the nest was, which progressed from cotton on the bottom of the nest, to a bowl nest, and finally to a dome nest.

2.3.4.2 Marble burying test

Nest-building behavior was used as a measure of the compulsive-like phenotype of the mice [50]. Male and female mice of all the strains were singly housed and provided with a pre-weighed cotton roll in the cage-top food hopper. After 24 h, the cotton roll was removed and weighed [17,18,19,71]. The total nesting score was defined as the amount of cotton used over the 24-h period. Generally, all the mice incorporated the cotton into their nest. The more cotton was used, the more elaborate the nest was, which progressed from cotton on the bottom of the nest, to a bowl nest, and finally to a dome nest.

2.3.5 Measuring anxiety-like behaviors

2.3.5.1 Open field test

Open field behavior was used to assess anxiety-like behavior of the mice [92]. BIG, SMALL and Control male and female mice were transported in their home cages, singly housed and placed on a rack outside the testing room just prior to testing. The open field apparatus

consisted of an open field arena (40 × 40 × 30 cm) with 16 10 × 10 cm squares marked on its floor. For testing, mice were placed in the central 4 squares of the field and allowed to explore the arena for 5 min. Entries into the central (20 × 20 cm) and peripheral squares were recorded [40] by the ANYMaze video-tracking program (Stoelting Co., IL, USA). The apparatus was cleaned before each test.

2.3.5.2 Elevated plus maze

Assessment of anxiety-like behavior was also determined with the elevated plus maze test [114]. The plus maze consisted of two open arms (5 × 40 cm) and two closed arms (5 × 40 × 20 cm) at right angles to each other. Each mouse was placed in the central square facing an open arm, and was allowed to explore the maze for 5 min. The time spent on the open arms was determined [115] by the ANYMaze video tracking program (Stoelting Co., IL, USA). An entry was defined as all four paws being on the arm. The maze was cleaned before each test.

2.3.6 Measuring novel object recognition behavior

The novel object recognition test was performed to measure object recognition memory [8]. During day 1, mice were habituated to the open field arena (40 × 40 × 30 cm) for 3 min. Twenty-four hours later on day 2, the time spent on investigating two identical objects (plastic toys) within a 5 cm distance in the open field arena was recorded for 3 min with the ANYMaze video tracking program (Stoelting.co). Mice were then taken out of the arena and returned to their home cages for 4 h. After 4 h one of the objects was replaced with a novel object of different shape and size and animals were then reintroduced into the arena and allowed to explore the objects for 3 min. Time spent exploring the familiar and novel objects were recorded. The preference of one object over another was assessed through the Recognition Index (RI) which is the time spent on the novel object relative to the time spent on both novel and familiar objects: [RI = TN/(TN + TF)] where TN is time spent on the novel object and TF is time spent on the familiar object) [38,67].

2.3.7 Measuring depression-like behavior

Time spent immobile in the forced swim test was used as a measure of depression-like behavior [41,90,91], where immobility is defined as the absence of no active behaviors like swimming, jumping or diving [12,41,91]. Mice were introduced in a glass cylinder, 20.5 cm in diameter and 21.5 cm in depth, which was filled with 18 cm of water maintained at 25–27 °C for 10 min [44].

2.3.8 Corticosterone ELISA

Mice were euthanized after the forced swim test through cervical dislocation to measure stress induced corticosterone levels. Trunk blood was collected randomly from each strain and sex (C1 males n = 4; C1 females n = 5; C2 males n = 6; C2 females n = 6; BIG1 males n = 4; BIG1 females n = 4; BIG2 males n = 7; BIG2 females n = 5; SML1 males n = 5; SML1 females n = 5; SML2 males n = 6; SML2 females n = 6) in chilled heparinized tubes and stored at −80 °C. After thawing the blood was centrifuged at 1000 ×g for 20 min. The plasma was extracted and placed in fresh tubes and stored at −80 °C. The corticosterone levels were determined using a corticosterone ELISA kit from Cayman Chemicals (Catalogue ID: 500,655) as per the manufacturer's instructions and as previously described [42]. The detection limit (80% B/B0) of the assay is approximately 80 pg/mL (Cayman) with inter- and intra-assay coefficient of variation of 2.2% and 8.9%, respectively

2.3.9 Statistical analyses

All measurement values are expressed in mean ± standard error of the mean (SEM). Statistical Analysis System (SAS Version 9.4, Cary, NC) software was used for statistical analyses. All behavioral and biochemical measures were tested in a general linear model (GLM) analysis of variance (ANOVA) for effects of strain (BIG, SMALL, Control), sex (female, males), replicate (1, 2) nested within strain, and strain x sex interaction. If significant effects were found, appropriate post hoc pair-wise comparisons were conducted using the Tukey's Studentized

Range. If the replicate effect was significant, the strain effect was tested over the replicate effect. If the replicate effect was not significant, the strain effect was tested over the error term. The total nesting score was square root transformed to obtain a more normal distribution [17, 18,19], while the data are presented as non-transformed nesting scores. Significant correlations between behaviors within strains were determined through linear regression.

2.4 Results

2.4.1 Behavioral data

2.4.1.1 Strain, replicate and sex differences in nest building

Significant strain ($F_{2,3} = 175.41$, $p < 0.001$), replicate ($F_{3,226} = 3.71$, $p < 0.02$) and sex ($F_{1,226} = 15.61$, $p < 0.0001$) effects were observed for compulsive-like nest building. Both male and female big nest builders (BIG1 and BIG2) used more cotton to build a nest than the Control mice (C1 and C2) and the SMALL mice (SML1 and SML2), and the Control mice built bigger nests than the SMALL mice. Most SML2 male mice did not use any cotton. The replicate effect was due to significant differences between the BIG1 and BIG2 males and C1 and C2 females. Sex differences were attributed to BIG1 and BIG2 males using more cotton than proestrus females for nesting (Fig 2.1). The significant interaction between strain and sex ($F_{2,226} = 16.68$, $p < 0.0001$) was due to females and males not being different in total nesting scores in the C1 and C2, and SML1 and SML2 strains, while the BIG strains showed large differences.

2.4.1.2 Replicate and sex differences in marble burying

For compulsive-like marble burying, the strain differences were marginally significant ($F_{2,3} = 9.02$, $p < 0.054$), predominantly due to the significant replicate effect ($F_{3,226} = 10.21$, $p < 0.0001$), although the general trend of BIG mice burying more marbles than SMALL mice with Control mice having intermediate values was in line with previously found significant strain differences [50]. The replicate effect was predominantly due to C1 females burying more

marbles than C2 females. The significant sex effect ($F_{1,226} = 11.03$, $p < 0.002$) resulted from C2 and BIG1 males burying more marbles than their female counterparts (Fig 2.2). The significant sex by strain interaction effect ($F_{2,226} = 4.07$, $p < 0.02$) was due to the SMALL strains not showing sex or replicate effects while the other strains did.

2.4.1.3 Replicate differences in open field behavior

There were no significant strain ($F_{2,3} = 1.16$, $p > 0.40$), sex ($F_{1,226} = 1.73$, $p > 0.15$) and strain by sex interaction ($F_{2,226} = 2.54$, $p = 0.08$) effects on anxiety-like behavior in the open field, as measured by the number of central entries. Significant replicate effects ($F_{3,226} = 17.87$, $p < 0.0001$) were due to female and male C2 mice making more central entries than C1 mice. Replicate effects were also due to female and male SML1 mice making more central entries than SML2 mice (Fig 2.3).

2.4.1.4 Strain differences in elevated plus maze behavior

In the elevated plus maze test, anxiety-like behavior, as measured by the time spent on the open arms, was significantly influenced by strain ($F_{2,226} = 22.11$, $p < 0.0001$) without a significant replicate effect ($F_{3,226} = 1.11$, $p > 0.30$). The strain differences were due to BIG2 males spending more time on the open arms than C2, SML1 and SML2 males and the BIG1 and C1 males spending more time on the open arms than the SML2 males. For females, the C1, C2, SML1 and SML2 strains spent less time on open arms than the BIG1 and BIG2 females. No significant sex ($F_{1,226} = 0.75$, $p > 0.30$) and strain by sex interaction ($F_{2,226} = 0.74$, $p > 0.40$) effects were found (Fig 2.4).

2.4.1.5 Sex and replicate differences in novel-object recognition memory

No significant strain ($F_{2,3} = 5.33$, $p > 0.09$) and strain by sex interaction ($F_{2,226} = 2.80$, $p > 0.05$) effects were found for novel object recognition. The significant effects of sex ($F_{1,226} = 6.87$, $p < 0.01$) on novel object recognition was mostly due to female C1 mice showing

better performance than their male counterparts (Fig 2.5). A significant replicate effect was found ($F_{3,226} = 3.44$, $p < 0.01$), which was predominantly due to differences between C1 and C2 males.

2.4.1.6 Sex and replicate differences in the forced swim test

In the forced swim test, depression-like behavior, as measured by duration of immobility, revealed no significant strain ($F_{2,3} = 1.78$, $p > 0.25$) and strain by sex interaction ($F_{2,226} = 1.82$, $p > 0.15$) effects. The significant sex ($F_{1,226} = 5.67$, $p < 0.05$) effect was due to proestrus females demonstrating less depression-like immobility behavior than did males, especially SML1, BIG1, C1, but not SML2 strains (Fig 2.6). The significant replicate effect ($F_{3,226} = 8.64$, $p < 0.0001$) was due to differences between the SML1 and SML2 males and the C1 and C2 females.

2.4.2 Strain differences in corticosterone plasma levels

Plasma corticosterone levels were significantly influenced by strain ($F_{2,52} = 12.17$, $p < 0.0001$) without a replicate effect ($F_{3,52} = 2.53$, $p > 0.05$). The BIG and SMALL nest-building strains generally mounted a higher corticosterone response to forced swim followed by euthanasia. Corticosterone levels in male BIG1 and SML1 strains were significantly higher than the C2 strain (Fig 2.7). Also, BIG1 males had higher corticosterone levels than C1 males. In females, both the BIG and SMALL strains had higher corticosterone levels when compared to the Control strains. No significant sex ($F_{3,52} = 3.68$, $p > 0.05$) and strain by sex interaction ($F_{2,52} = 1.54$, $p > 0.20$) effects were observed.

2.5 Discussion

In this study we report for the first time that females of both the BIG strains displayed face validity as a non-induced compulsive-like model by using more cotton for nest building and burying more marbles compared to the females of the SMALL strains. For nest-building behavior, the female Control strains were intermediate but closer to the SMALL strains, while for

marble burying one of the Control strains was similar to the BIG strains and the other Control strain was similar to the SMALL strains. BIG1 and BIG2 females had less compulsive-like nesting (48% and 28%, respectively), when compared to BIG1 and BIG2 males establishing a sex difference, which was specific to the compulsive-like mice because the SMALL and Control strains did not show differences between males and females. We hypothesize that this difference was due to using proestrus females in this study with higher estrogen levels. For marble burying a similar sex difference was also observed for the BIG1 strain, but not the BIG2 strain although the trend was in the same direction.

Prior studies have shown that compulsive-like behaviors were enhanced in the absence of estrogen, such as estrogen-deficient male mice that showed development of compulsive-like wheel running and grooming behavior. These behaviors were considerably reduced with estrogen replacement [58]. Estrogen administration in ovariectomized rats with 8-OH-DPAT induced compulsive-like traits caused significant reduction in the compulsive-like behavior [33]. Females in proestrus have high physiological circulating estrogen levels [22,99], but whether estrogen played a direct role in reducing compulsive-like behaviors in proestrus female compared to male compulsive-like mice remains to be elucidated. Cohort based clinical studies have found that overall males have higher vulnerability to obsessions and compulsions when compared to females [14,53]. Although obsessions are difficult to model in animals, the severity of compulsions can be a suitable measure of it [34]. Therefore, the higher levels of compulsive-like behaviors in BIG males compared to BIG females may further add face validity to the mouse model for understanding sex differences that attribute to the complexity of OCD.

Open field and elevated plus maze tests were used to assess anxiety-like behavior in males and females of BIG, SMALL and Control strains. No significant strain effects were found for the number of central entries in the open field, which indicates that this measure of anxiety-like behavior did not correlate with the level of compulsive-like behavior. However, significant

strain differences were observed in elevated plus maze tests for both males and females. Generally the BIG strains showed less anxiety-like behavior in the elevated plus maze test when compared to the SMALL strains, which is consistent with our previous findings [50], and the females showed this general pattern most clearly. The responses of the six strains in the open field and elevated plus maze varied, which may be due to these tests measuring different aspects of emotionality associated with anxiety [6,93].

Although BIG mice overall were less anxious than the SMALL mice in the elevated plus maze, within each BIG strain the correlations were generally opposite. Elevated plus maze exploration had a negative correlation with levels of compulsive-like nesting ($r = -0.708$, $p < 0.005$) and marble burying ($r = -0.562$, $p < 0.05$) behavior in BIG2 females indicating that an increase in compulsive-like behavior was associated with more anxiety (less exploration of the open arms). A negative correlation was also found between marble burying (but not nesting) with open arm exploration in BIG1 males ($r = -0.817$, $p < 0.0001$). This result could be due to a changing genetic correlation structure as selection progressed, similar to what we found in these mice for the relationship between food consumption and nest building previously [19]. Alternatively, genetic drift and founder effects [17,19] might be responsible for the correlations observed in just the BIG2 females and the BIG1 males.

The association of OCD with general anxiety is deemed controversial [103]. Studies have shown that the ego-dystonic and intrusive nature of obsessions differ largely from general anxiety [64,65]. There is also large heterogeneity and variability of anxiety in OCD, though general anxiety is a common comorbid psychiatric condition associated with the disorder [5,84]. This makes it an ambiguous indicator for the disorder [85]. General anxiety and panic disorders have a late onset when compared to OCD, which has a very early onset [95]. In addition, neurocircuitry models proposed for anxiety and OCD differ. While anxiety and panic disorders have been linked to dysregulation of amygdala-ventromedial prefrontal cortex-hippocampus

circuitry [98], OCD is thought to occur due to abnormal fronto-striatal circuitry [36,49,88,94]. Consequently, findings from the current study will allow the BIG2 female and BIG1 male strains to be used for studying association between various forms of compulsivity and anxiety. The BIG1 females and BIG2 males on the other hand can provide understanding of various forms of compulsivity and how they vary based on sex and genetic background.

In the depression-like forced swim test, no significant relationship was found between the level of compulsive-like behavior and immobility times as the strain effect was not significant. However, significant sex differences were observed with the females generally showing less depression-like behavior than males. Rodent studies have shown sex and strain differences in depression-like behavior in the forced swim task [13,102]. Proestrus female mice and rats with higher circulating estrogen have been found to be less immobile in forced swim task when compared to the male counterparts [43,44,47], which is similar to our findings. Depression is a common comorbidity associated with OCD [5, 87,104], but has been found to vary considerably among studies [3]. Human studies have shown that anxiety and depressions are influenced by factors like race/ethnicity and gender differences [78]. Hence, an effect of genetic background coupled with sex differences could influence the comorbid depression disorders in OCD and present a complex set of interactions, also reflected in our six mouse strains with different compulsive-like phenotypes.

The novel object recognition test revealed no significant strain effects, although the Control strains tended to outperform the BIG and SMALL strains. Females significantly outperformed the males, mostly due to the Control strains and less so due to the SMALL strains. The novel object recognition task is useful for studying working memory [38,46,100]. Several clinical studies have reported deficits in working memory among OCD patients [75,81,110], while others have reported no significant difference [85,86]. Our findings in the mice may be due to the fact that differences in working memory capacity among OCD patients are

linked to intrusive thoughts or obsessions [15], which simply cannot be assessed in animals. Alternatively, novel object recognition memory may not be equivalent to the working memory deficits measured in OCD patients

The female Control mice had significantly lower plasma corticosterone levels than the BIG or SMALL strain females. This general pattern was also observed in the males, but to a lesser degree. Previous studies have shown that female rodents secrete higher levels of corticosterone than males [10,21,66], which the BIG1, BIG2, and SML2 mice tended to show as well, although the overall sex effect was not significant. Additionally, in proestrus, corticosterone levels in response to stress are higher [9,21,35,112]. Coping with stress is promoted by the hypothalamic-pituitary-adrenal (HPA) axis [28,106] and corticosterone is a primary end point of HPA axis activation in mice [54]. OCD is known to be stress responsive since symptoms not only increase during periods of stress but stressful events can also precede the onset of obsessive-compulsive symptoms [80]. Our results show that selection to behavioral extremes, both in the high and low direction of nesting, resulted in enhanced activation of the HPA axis due to stress imparted by the forced swim test. OCD patients have higher baseline urine [45] and blood [61] cortisol levels than healthy matched controls, which can be compared to the higher stress-induced corticosterone levels in the BIG strains compared to the Control strains.

Significant replicate effects nested within strain were found for nest building, marble burying, open field behavior, novel object recognition memory, and the forced swim test. These replicate effects were most likely due to differences in genetic background as a result of random genetic drift and founder effects within each strain [17,19]. Of special interest are the replicate effects between the BIG strains as they represent the compulsive-like phenotype and may correspond to subtypes of compulsive-like phenotypes as seen in human OCD patients [39,48,68]. The male BIG1 strain nested more than the BIG2 strain, while the BIG2 strain showed more anxiety-like behavior in the elevated plus maze than the BIG1 strain. However,

the females were not different from each other for either trait indicating a potential sex by genotype interaction. Further, sex differences in only the BIG1 but not the BIG2 strains in marble burying add heterogeneity based on specific compulsive-like traits that might significantly vary depending on the sex and genotype.

2.6 Conclusion

In the current study, we confirmed that females of the compulsive-like BIG strains also have face validity as a mouse model of OCD, enhancing our earlier findings in males [50]. Proestrus females also showed lower levels of compulsive-like behaviors than males, which suggest that physiological changes in females related to the estrous cycle might influence compulsive-like behavioral expression. A differential response was observed in anxiety-like behaviors with replicate effects in the open field and strain differences in the elevated plus maze. Sex differences were seen for depression-like behavior with an elevated HPA axis response in females compared to males. Overall, our mouse strains can be used to better understand how genetic and sex factors and behavioral correlates contribute to the compulsive-like phenotype. Replicate effects in compulsive-like expression also indicate symptom heterogeneity that is strongly associated with OCD making it a complex neuropsychiatric disorder. Future studies will aim at investigating sex and strain differences in first line therapy responses and the neurobiological mechanisms in the mouse model that could broaden the understanding of heterogeneity and drug unresponsiveness in certain OCD subtypes when compared to others.

2.7 Acknowledgements

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2.8 Figures

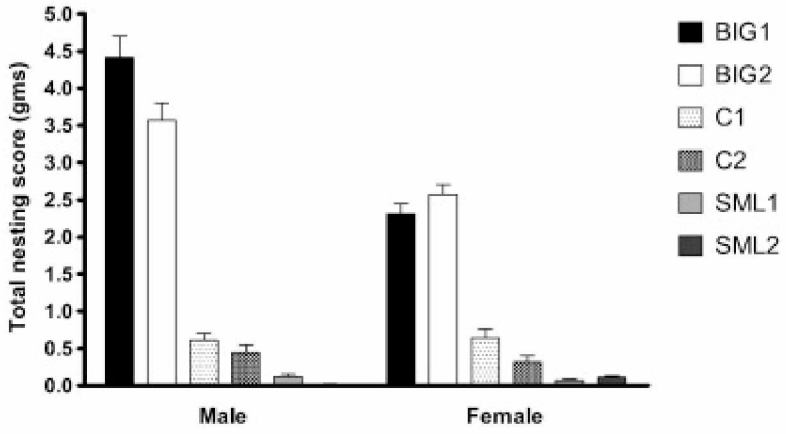

Fig 2.1 Nest-building behavior.

Mean (± SEM) total nesting scores of male and female BIG, SMALL and Control replicate strains. BIG1 and BIG2 males had higher nesting score than C1 ($t = 15.28$ $p < 0.001$ and $t = 12.95$ $p < 0.001$), C2 ($t = 17.20$ $p < 0.001$ and $t = 14.86$ $p < 0.001$), SML1 ($t = 20.47$ $p < 0.001$ and $t = 18.16$ $p < 0.001$) and SML2 ($t = 22.72$ $p < 0.001$ and $t = 20.42$ $p < 0.001$) males. BIG1 males had higher nesting score than BIG2 males ($t = 2.365$ $p < 0.05$). BIG1 and BIG2 females had higher nesting score than C1 ($t = 9.564$ $p < 0.001$ and $t = 9.364$ $p < 0.001$), C2 ($t = 11.81$ $p < 0.001$ and $t = 11.43$ $p < 0.001$), SML1 ($t = 15.19$ $p < 0.001$ and $t = 14.34$ $p < 0.001$) and SML2 ($t = 14.20$ $p < 0.001$ and $t = 13.49$ $p < 0.001$) females. BIG1 and BIG2 males had higher nesting score than BIG1 and BIG2 females ($t = 6.682$ $p < 0.001$ and $t = 2.886$ $p < 0.05$).

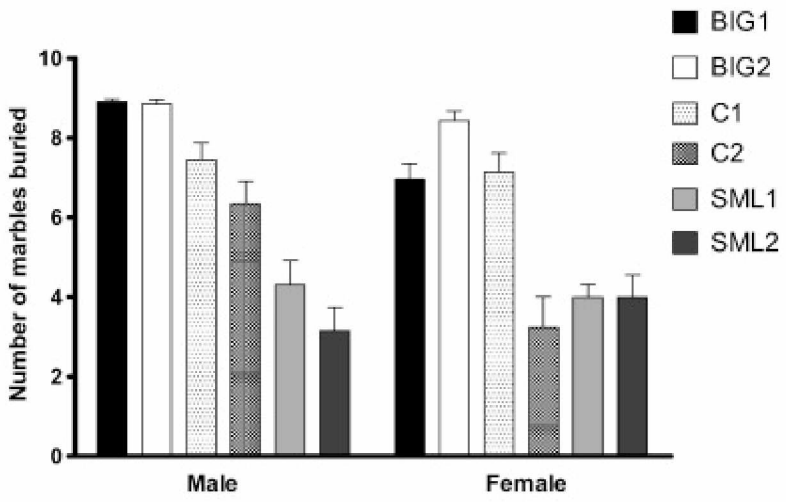

Fig 2.2 Marble burying behavior.

Mean (± SEM) number of marbles buried in male and female BIG, SMALL and Control replicate strains. Replicate effect was due to C1 females burying more marbles than C2 females ($t = 5.850$ $p < 0.001$). BIG1 and C2 males buried more marbles than BIG1 ($t = 3.132$ $p < 0.05$) and C2 ($t = 4.201$ $p < 0.001$) females, contributing to the sex differences and strain by sex interactions.

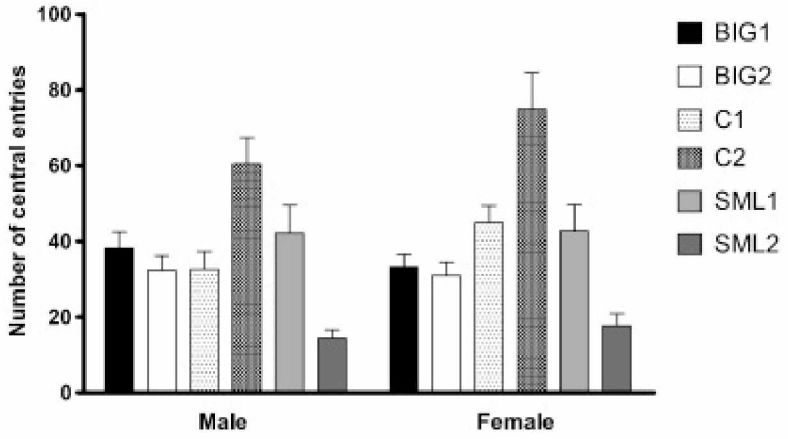

Fig 2.3 Open field behavior.

Mean (± SEM) number of central entries in male and female BIG, SMALL and Control replicate strains. C2 males and females had more central entries than C1 males ($t = 3.739$ $p < 0.001$) and females ($t = 3.903$ $p < 0.001$), respectively. SML1 males and females had more central entries than SML2 males ($t = 3.624$ $p < 0.001$) and females ($t = 3.903$ $p < 0.01$), respectively.

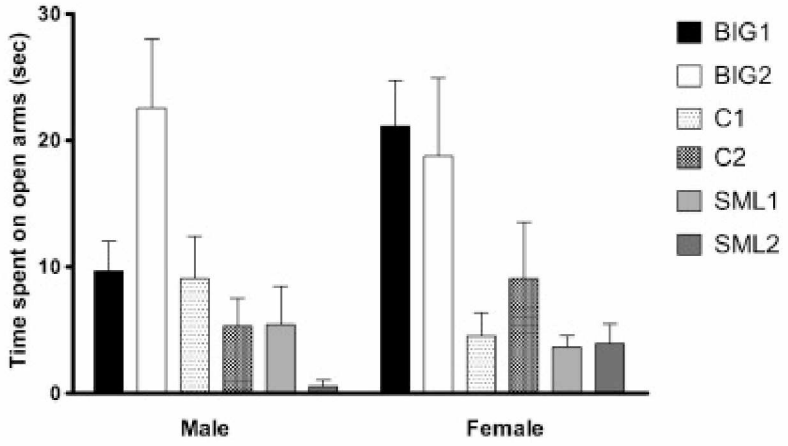

Fig 2.4 Elevated plus maze behavior.

Mean (± SEM) time spent on open arms of male and female BIG, SMALL and Control replicate strains. Significant strain differences were observed in time spent on open arms between male BIG2 with C2 ($t = 3.881$, $p < 0.001$), SML1 ($t = 3.807$, $p < 0.001$) and SML2 strains ($t = 4.899$, $p < 0.001$). The C1, C2, SML1 and SML2 females spent less time on open arms when compared to BIG1 females ($t = 3.836$, $p < 0.001$; $t = 2.627$, $p < 0.05$; $t = 4.041$, $p < 0.001$; $t = 3.923$, $p < 0.001$, respectively). The C1, SML1 and SML2 females spent less time on open arms when compared to BIG2 females ($t = 2.904$, $p < 0.01$; $t = 3.085$, $p < 0.01$; $t = 2.994$, $p < 0.01$, respectively).

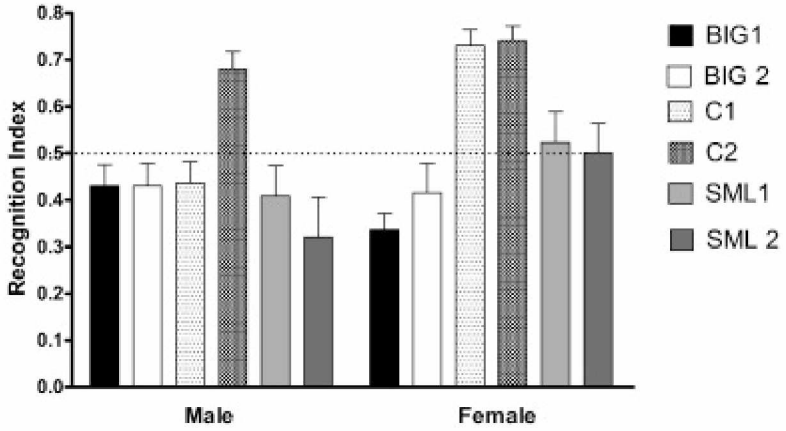

Fig 2.5 Novel object recognition behavior.

Mean (± SEM) recognition index of male and female BIG, SMALL and Control replicate strains. The C2 males had higher RI values when compared to C1 males ($t = 3.340$, $p < 0.01$). The C1 females had higher RI when compared to C1 males ($t = 3.921$, $p < 0.001$).

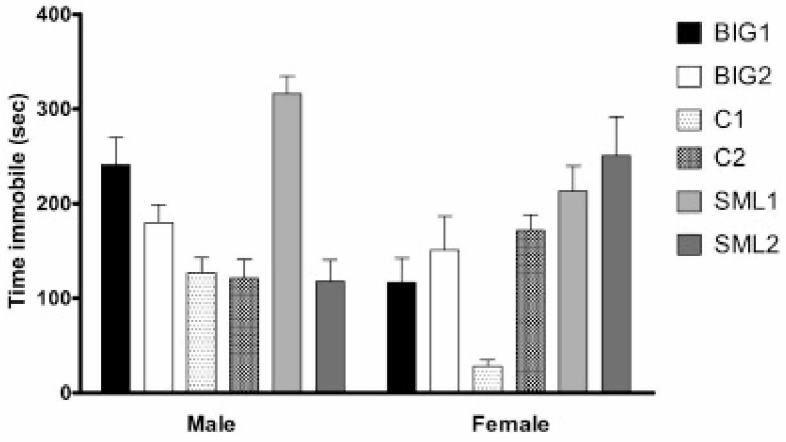

Fig 2.6 Forced swim test.

Mean (± SEM) immobility time of male and female BIG, SMALL and Control replicate strains. SML1 males had higher immobility times than SML2 males ($t = 5.796$, $p < 0.001$) while C2 females had higher immobility times than C1 females ($t = 4.229$, $p < 0.001$). BIG1, C1 and SML1 males had higher immobility times when compared to BIG1 ($t = 3.063$, $p < 0.05$), C1 ($t = 3.778$, $p < 0.01$) and SML1 ($t = 3.083$, $p < 0.01$) females. In the SML2 strain, females had higher immobility time than the males ($t = 3.827$, $p < 0.01$).

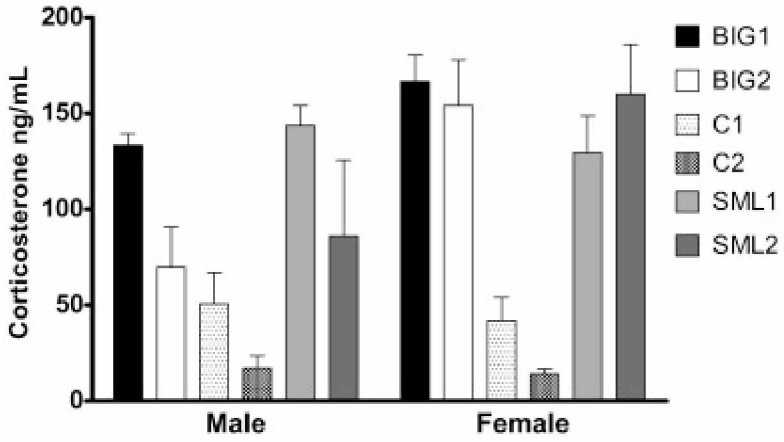

Fig 2.7 Plasma corticosterone levels.

Mean (± SEM) corticosterone levels as a measure of HPA axis stress response of male and female BIG, SMALL and Control replicate strains. Levels in male BIG1 and SML1 strains were significantly higher than the Control C2 strain ($t = 3.773$, $p < 0.001$ and $t = 4.385$, $p < 0.001$, respectively). BIG1 ($t = 2.455$, $p < 0.05$) and SML1 ($t = 2.916$, $p < 0.05$) males had higher corticosterone levels than C1 males. Female BIG1, BIG2, SML1 and SML2 also had higher corticosterone levels than female C1 ($t = 3.90$, $p < 0.001$; $t = 3.73$, $p < 0.001$; $t = 2.91$, $p < 0.05$ and $t = 4.91$, $p < 0.001$, respectively) and C2 ($t = 4.95$, $p < 0.001$; $t = 4.85$, $p < 0.001$; $t = 3.99$, $p < 0.001$ and $t = 5.30$, $p < 0.001$, respectively).

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Chapter 3: Strain and trait specific attenuation of compulsive-like behavior by fluvoxamine in a non-induced mouse model of obsessive-compulsive disorder.[2]

3.1 Abstract

The influence of genetic background on efficacy of drug treatment in OCD is poorly understood. The current study evaluates the role of strain and compulsive trait differences in response to fluvoxamine, a common OCD drug, in two different mouse strains (BIG1 and BIG2) exhibiting a spontaneous compulsive-like phenotype. For compulsive-like nest-building behavior, dose-dependent attenuation of nesting was observed for BIG1 compulsive-like strain following one hour of administration. No significant differences were observed for BIG2 strain after one hour. Fluvoxamine dose dependently decreased the number of marbles buried in both strains one hour after administration. For anxiety-like behaviors in the open field no significant drug effects were found for the latency to leave the center and the number of line crossings. The 10 mg/kg fluvoxamine dose increased time in the center for the BIG2 strain compared to the 0 mg/kg control group. Taken together these results suggest a strong role of strain and specific compulsive traits in influencing efficacy of fluvoxamine. The results also indicate better effectiveness of intermediate but not high doses of fluvoxamine in mitigating certain anxiety-like behaviors in the compulsive-like condition.The data from this study corroborates the differential treatment responses in human OCD patients, further validating the use of our compulsive-like mouse strains for understanding the neurobiology associated with the clinical heterogeneity in OCD.

[2] Submitted as [2]Mitra S, Bult-Ito A. Strain and trait specific attenuation of compulsive-like behavior by fluvoxamine in a non-induced mouse model of obsessive-compulsive disorder. Journal of Behavioral Pharmacology.

3.2 Introduction

Obsessive-compulsive disorder (OCD) is an intricate heterogeneous disorder characterized by persistent obsessions (thoughts) and compulsions (repetitive behaviors) (Karno et al., 1988; Albert et al., 2013). In the United States OCD has an estimated lifetime occurrence of about 2.3% (Ruscio et al., 2010). The social functioning, relationships, quality of life and socio-economic status of patients suffering from OCD are significantly impacted (Fontenelle et al., 2010; Hollander et al., 2010).

The most common forms of first line treatments for OCD are selective serotonin reuptake inhibitors (SSRI) and cognitive behavioral therapy (Kellner, 2010; Pittenger et al., 2005; Bandelow et al., 2012). Fluvoxamine is a first generation SSRI widely used for treatment of OCD (Irons, 2005). It is a potent inhibitor of serotonin reuptake at the synapses and has no effect on reuptake of dopamine and nor-epinephrine (Goodman et al., 1997). Many clinical studies have shown efficacy of fluvoxamine in treating OCD (Goodman et al., 1989, Mallya et al., 1992; Milanfranchi et al., 1997; Mundo et al., 2001). However, 40-60% of the OCD patients are refractory to the first line treatments (Nakamae, 2013). Even patients categorized as clinical responders continue exhibiting impairments from their residual obsessions and compulsions (Lack, 2012). Considering such high drug resistant rates among patients, a better understanding of the efficacy of first line treatments on specific compulsive traits in OCD subjects of different genetic backgrounds is needed.

Investigating drug intervention in mouse strains exhibiting spontaneous compulsive-like behaviors can be a valuable starting point for determining if there is any role of genetic background and compulsive traits in influencing drug response. OCD has a strong genetic connection which has been established in both human and rodent studies (Hettema et al., 2001; Pato et al., 2002; Welch et al., 2007; Wang et al.,2010; Ting and Feng, 2011; Taylor, 2013). According to Maio et al. (2014), comparing our compulsive-like strains can be useful for

understanding the disease mechanism during the OCD condition. As per our knowledge, no studies have been undertaken to elucidate the efficacy of a first line OCD treatment on two different spontaneously compulsive-like mouse strains for compulsive- and anxiety-like behaviors. Hence, in the current study we aim to investigate the efficacy of fluvoxamine in attenuating two separate compulsive-like behaviors (marble burying and nest-building) exhibited by the compulsive-like BIG mice (Greene-Schloesser et al., 2011) and anxiety-like behavior (open field).

The current mouse model was developed by bidirectionally selecting house mice, Mus musculus, for nest-building behavior (Lynch, 1980; Bult and Lynch, 2000). The stock population for the original selection experiment (Lynch, 1980) was a cross among eight inbred strains yielding the HS/Ibg outbred strain (McClearn et al., 1970; Lynch, 1980). Nest-building behavior, which is homologous to hoarding in humans with OCD (Warneke, 1993), is considered a measure of compulsive-like behavior in mice (Greene- Schloesser et al., 2011). Bidirectional selection resulted in three main strains of mice exhibiting three different levels of nesting behavior. The BIG strains consistently display high levels of nesting with a forty-fold difference in the amount of cotton used (typically the BIG1 and BIG2 mice nest on an average 8 grams of cotton in 24-hour period which is in contrast to 0.20 grams for non-compulsive SMALL and 0.70 grams for Control mice) when compared to the SMALL strains which display very low levels of nesting. The randomly-bred Control strains serve as a selection control and show intermediate levels of nesting (Bult and Lynch, 2000). Each of these BIG, SMALL and Control strains has two replicate strains (BIG1 and BIG2, SML1 and SML2, and C1 and C2). The BIG strains of mice exhibit compulsive-like behavior (nest-building and marble burying) without gene manipulations, behavioral inductions, or administration of psychostimulants, which makes them a novel non-induced model of OCD (Greene- Schloesser et al., 2011). Further, consistent differences in compulsive- and anxiety-like behaviors have also been observed between the two BIG strains

(BIG1 and BIG2) making them ideal for investigating the role of strain differences in influencing drug effectiveness.

3.3 Methods

3.3.1 Animals

Intact compulsive-like BIG male house mouse (Mus musculus) strains (BIG1, BIG2) were used for the study (BIG1 n= 36: BIG2 n= 38). A total of 12 animals per group per strain was initially considered but 12 mice from BIG1 and 10 mice from BIG2 strain was euthanized due to skin scabbing). Mice were raised in polypropylene cages (27×17×12 cm) with wood shavings under a 12-12 light-dark cycle at 22±1°C. Food (Purina Mills, Lab Diet Mouse Diet #5015, St. Louis, MO) and water were provided ad libitum. Pups were weaned at 19-21 days of age and housed with same-sex littermates. All animals were approximately 60 days old at the start of the experiment.

3.3.2 Drug administration

Fluvoxamine maleate (Sigma Aldrich) was dissolved in physiological saline (pH=7.4) to yield final doses of 10 mg/kg, 20 mg/kg and 40 mg/kg (pH 7-7.4). Saline served as a vehicle (0 mg/kg) control. All male compulsive-like mice were divided into four treatment groups (starting at 12 animals per group) comprising vehicle (sterile saline with pH adjusted to 7.4), 10 mg/kg, 20 mg/kg and 40 mg/kg fluvoxamine. Animals in each group received subcutaneous injections of fluvoxamine/vehicle daily for 17 days.

3.3.3 Experimental design

All Animals underwent behavioral testing consecutively consisting of nest building on day 15, marble burying on day 16 and open field on day 17 of the 17-day fluvoxamine treatment regimen. The sequence of behavioral tests, i.e., doing the anxiety-like tests before the

compulsive-like tests or vice versa, does not affect the performance in any of these behaviors in our mouse strains (data not shown).

For nest-building, data was collected during the first hour after fluvoxamine administration. Marble burying and open field were performed one hour after drug administration. Marble burying was performed for 15 minutes and open field for 3 minutes (Greene-Schloesser et al., 2011). Testing was performed in the light phase of the light-dark cycle and the University of Alaska Fairbanks Institutional Animal Care and Use Committee approved the animal care and experimental procedures (IACUC assurance number 718349).

3.3.4 Compulsive-like nest-building behavior

Nest-building behavior was used as a measure of the compulsive-like phenotype of the mice (Greene-Schloesser et al., 2011). Male mice of both the BIG strains (BIG1 andBIG2) were singly housed and provided with a pre-weighed cotton roll in the cage-top food hopper. The cotton roll was removed and weighed (Bult and Lynch, 1996; Bult and Lynch, 1997; Bult and Lynch, 2000) after 1 hour of fluvoxamine administration. The total nesting score was defined as the amount of cotton used over a 1-hour period.

3.3.5 Compulsive-like marble burying behavior

The marble-burying test was also used for determining compulsive-like behavior

(Takeuchi et al., 2002; Thomas et al., 2009; Greene-Schloesser et al., 2011; Angoa-Perez et al., 2013). One hour after fluvoxamine administration, compulsive-like male mice were individually introduced into a polypropylene cage (37×21×14 cm) containing 20 glass marbles (10 mm in diameter) spaced evenly on wood chip bedding 5 cm deep without access to food or water for 15 min (Greene-Schloesser et al., 2011). Testing was carried out in the testing room separate from the housing room. The total number of marbles buried at least 2/3 in the 15-min period was

quantified as compulsive-like digging behavior. After the 15-min test, the animals were returned to their home cages.

3.3.6 Anxiety-like open field behavior

Anxiety-like behavior in the mice was evaluated through open field test (Simon et al., 1994; Prut and Belzung, 2003). BIG1 and BIG2 males were transported in their home cages, singly housed and placed on a rack outside the testing room just prior to beginning testing. The open field apparatus consisted of an open field arena (40 x 40 x 30 cm) with 16 10 x 10 cm squares on its floor. For testing, animals were placed in the center of the field and allowed to explore the arena for 3 minutes. Latency to leave the central square (20 x 20 cm) initially and entries into the central zone (20 x 20 cm) after leaving it initially were recorded to quantify anxiety like behaviors. The total number of line crossings was recorded as a measure of locomotor activity (Frye et al., 2006; Nosek et al., 2008; Greene-Schloesser et al., 2011). The apparatus was cleaned before each test.

3.3.7 Statistical analysis

Values from all behavioral assessments were expressed in mean ± standard error of the mean (SEM). Statistical Analysis System Software (Version 9.4, Cary, NC) was used for statistical analyses. All behavioral measures were tested in a general linear model (GLM) analysis of variance (ANOVA) for effects of strain, drug and strain x drug interaction effects. If significant effects were found, appropriate post-hoc pair-wise comparisons were conducted using the Tukey's Studentized Range test. The total nesting score was square-root transformed to obtain a more normal distribution (Bult and Lynch, 2000), while the data are presented as non-transformed nesting scores.

3.4 Results

3.4.1 Strain dependent attenuation of compulsive-like nesting behavior by fluvoxamine

After 1 hour of fluvoxamine administration there was a significant drug effect ($F_{3,74}$ = 11.73, $p<0.0001$). 20 and 40 mg/kg doses significantly attenuated the nesting scores for BIG1 strain when compared to vehicle (0 mg/kg). There was no significant effect of fluvoxamine on nesting in the compulsive-like BIG2 strain (Fig 3.1).

3.4.2 Dose-dependent attenuation of compulsive-like marble burying behavior by fluvoxamine in both the BIG strains.

A significant dose-dependent drug effect ($F_{3,74}$ = 13.08, $p<0.0001$) was observed in the number of marbles buried. The 20 and 40 mg/kg doses showed significant attenuation of marble burying when compared to vehicle in both the BIG1 and BIG2 strains. No significant strain ($F_{1,74}$ = 1.99, $p>0.16$) and drug by strain interaction ($F_{3,74}$ = 0.88, $p>0.45$) effects were found (Fig 3.2).

3.4.3 Significant strain differences in anxiety-like open field behavior

For anxiety-like open field test a significant strain effect ($F_{1,74}$ = 39.83 $p<0.0001$) for the latency to leave the central square was found with the BIG1 strain taking less time when compared to the BIG2 strain. The drug ($F_{3,74}$ = 2.21, $p>0.09$) and drug by strain interaction ($F_{3,74}$ = 0.76, $p>0.451$) effects were not significant (Fig 3.3a). For the time spent in the central square the strain effect was marginally significant ($F_{1,74}$ = 3.73, $p>0.05$) with the BIG1 mice tending to spend more time in the central square compared to the BIG2 mice. The significant drug effect ($F_{3,74}$ = 5.12, $p<0.003$) was primarily due to the 10 mg/kg dose which increased the time spent in the central zone for BIG2 strains compared to the vehicle (Fig 3.3b). The drug by strain interaction effect was not significant ($F_{3,74}$ = 0.47, $p>0.70$). For the number of line crossings there was a significant strain effect ($F_{1,74}$ = 19.61,$p<0.0001$), which was due to the BIG1 strains

having more line crossings when compared to the BIG2 strains (Fig 3.3c). No significant drug ($F_{3,74} = 0.77$, $p>0.51$) or drug by strain interaction ($F_{3,74} = 0.19$, $p>0.89$) effects were found.

3.5 Discussion

Our results showed that a fluvoxamine treatment regimen for 15-17 days resulted in significant dose-dependent attenuation of compulsive-like nesting in BIG1 but not BIG2 male mice. This effect was observed following one hour of drug administration on the day of testing. Marble burying behavior on the other hand was dose-dependently reduced by fluvoxamine in both BIG1 and BIG2 strains. The dose response is in congruence with human studies where high doses of fluvoxamine have been found to be more effective for compulsive behaviors when compared to low doses (Fontenelle et al., 2007; Ordacgi et al., 2009; Koran et al., 2010). Previous study with the BIG mice has shown that a two week (14 days) fluoxetine (SSRI) treatment significantly attenuates nesting and marble burying behavior (Greene-Schloesser et al., 2011) in the BIG1 strain. This is the first time we used both the BIG strains (BIG1 & BIG2) for comparing the compulsive-like behavioral response to a first line treatment, fluvoxamine. The results support both nesting and marble burying behavior for compulsive-like assessment in our model. This is in contrast to the compulsive-like deer mouse model with spontaneous stereotypy, in which marble burying behavior was not associated with stereotypy and did not respond to oral escitalopram treatment (Wolmarans et al., 2016b). So for deer mice, marble burying behavior did not appear to be a compulsive-like behavior. Interestingly, stereotypy and nest-building behavior also did not correlate, but 30% of the deer mice, irrespective of these two behaviors, showed unusually large nest building behavior, which was attenuated by escitalopram (Wolmarans et al., 2016a). So individual deer mice show different compulsive-like behaviors, i.e., stereotypy or nest building, but these were not correlated, while our BIG nest builders have a predictable compulsive-like phenotype showing both compulsive-like nesting and marble burying that can be attenuated with drugs used in OCD (Greene-Schloesser et al.,

2011) as shown in this study. Therefore, it appears that compulsive-like behaviors are specific and that not all compulsive-like behaviors are genetically correlated or affected by the environment in similar ways.

Locomotor activity was measured in the open field test (Hale et al., 2008) and was not affected by fluvoxamine. This result replicates another study with fluvoxamine in mice in which repeated fluvoxamine administration attenuated marble burying but not locomotion or exploratory behavior (Ichimaru et al., 1995). Overall, BIG2 mice showed larger levels of anxiety-like behaviors than BIG1 mice measured as time to leave the central square in the open field. This replicates earlier findings in these compulsive-like strains. For time spent in center in the BIG2 mice the 10 mg/kg group showed anxiolytic behavior when compared to the 0 mg/kg group, and the BIG1 strain showed the same trend. This effect was however not observed for the higher doses (20 and 40 mg/kg) for both the BIG1 strains, showing that fluvoxamine only had a moderate effect overall. This is an interesting finding since a prior study with mice has shown that fluvoxamine had no anxiolytic effects on different anxiety tests (Ichimaru et al., 1995). Our results show that behavioral response to fluvoxamine in our compulsive-like mice for anxiety-like measures could be very specific to assessed parameters (line crossings, time in center, latency to leave the center) and can vary between strains. While, fluvoxamine is well tolerated and used widely in the treatment of general anxiety disorder and OCD (Freeman et al., 1994; Irons, 2005; Mundo et al., 2000), not many studies have focused on looking at the effect of fluvoxamine in treating co-morbid anxiety in complex neuropsychiatric disorders (Nadeau et al., 2011). A study on the efficacy of fluvoxamine in treating autism spectrum disorder (ASD) and associated anxiety resulted in a poor response to treatment (Martin et al., 2003). The ego-dystonic and intrusive nature of obsessions differs largely from general anxiety (Langlois et al., 2000, 2000a). In addition to large heterogeneity and variability of anxiety in OCD, this makes it a non-significant indicator for the disorder (Nutt and Malizia, 2006). General anxiety disorders and

panic disorders have a late onset when compared to OCD, which has a very early onset (Rosenbaum et al., 1997). In addition, neurocircuitry models proposed for anxiety and OCD differ. While anxiety and panic disorders have been linked to dysregulation of amygdala-ventromedial prefrontal cortex-hippocampus circuitry (Rauch et al., 2006; Shin and Liberzon, 2010), OCD is thought to occur due to abnormal fronto-striatal circuitry (Fitzgerald et al., 2005; Rauch et al., 2007; Greenberg et al., 2010). A study from our lab on compulsive-like (BIG) and non-compulsive-like (SMALL) mice indicated that general anxiety appears to be separate from anxiety pertaining to compulsions (Greene-Schloesser et al., 2011). This is corroborated by other studies where association of OCD with general anxiety is deemed controversial (Stein et al., 2010).

There is currently a lack of consensus on the best treatment option for OCD patients (Pittenger et al., 2005). Significant relapse rates have been reported in clinical studies with SSRI's (Abramowitz et al., 2009). Medication differences have also been observed with these first line treatments. One such example is that of comorbid tics in children with OCD which reduces efficacy of SSRI (March et al., 2007). It is however unclear if the same effect is persistent in adults (Lack, 2012). Among various factors, compulsive traits and associated co-morbid symptoms can result in non responsiveness to treatments in the OCD condition (Koran, 2004). Studies have attributed strong genetic link to the etiological heterogeneity of OCD (Nestadt et al., 2000; Pauls, 2010). These genes have also been thought to contribute to the disease severity and treatment resistance (Ozaki et al., 2003; Wendland et al., 2008). Quantitative genetic analysis has indicated that nesting behavior is a highly polygenic trait (Bult and Lynch, 2000) which is consistent with OCD in humans likely influenced by many candidate genes (Pauls et al., 1999; Hettema et al., 2001; Stein, 2002; Smoller., 2009). Hence, our mouse model selected for varied levels of compulsive-like behavior, i.e., nest-building, provides a

critical insight into the role of genetic background and trait in influencing first line treatment efficacy.

3.6 Conclusion

This study adds predictive validity to our mouse model for investigating trait specific drug resistance OCD subtypes and the role of compulsive trait and genetic background in influencing drug action. Fluvoxamine commonly used to treat OCD, dose-dependently decreased nest building behavior in one compulsive-like strain and dose-dependently attenuated marble burying in both compulsive-like strains. This behavior and strain specific response, coupled with one of the compulsive-like strains being more anxious than the other, adds heterogeneity to our mouse model, similar to the heterogeneity seen in OCD. Future studies will involve probing into the mechanisms of trait specific drug resistance in the compulsive-like mouse strains.

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3.8 Figures

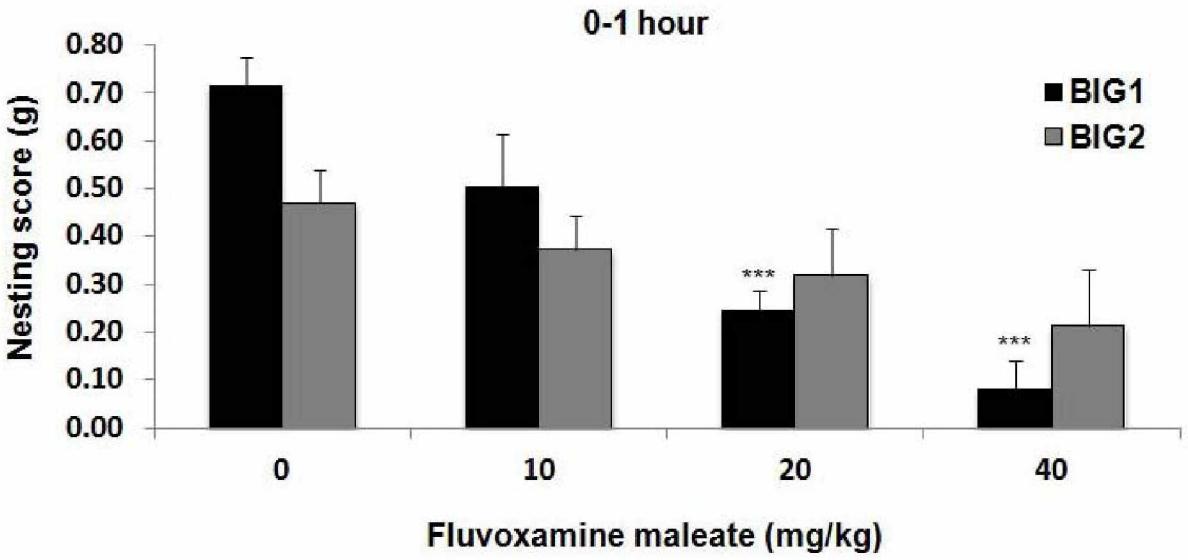


Fig 3.1 Compulsive-like nest-building behavior of the BIG1 and BIG2 strains.

The data represent the mean (± SEM) for the nesting score in grams for the 0-1 hour period. ***($p<0.0001$) indicates significant drug effect between 20 and 40 mg/kg with vehicle (0 mg/kg) in BIG1 strain. 0 mg/kg, n=11 and n=11; 10 mg/kg, n=7 and n=12; 20 mg/kg, n=10 and n=8; 40 mg/kg, n=6 and n=9 for BIG1 and BIG2, respectively.

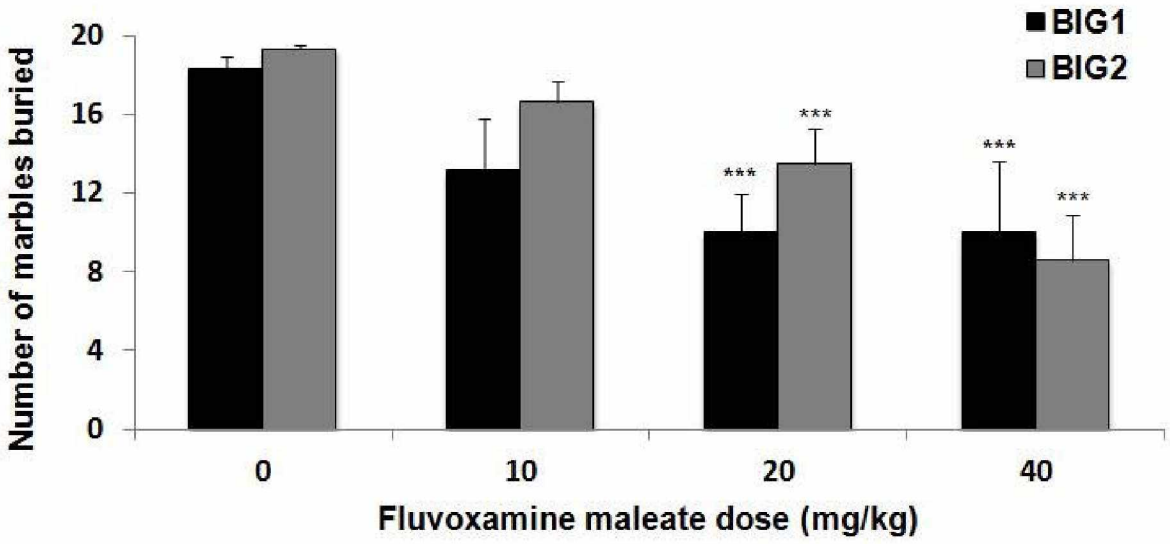
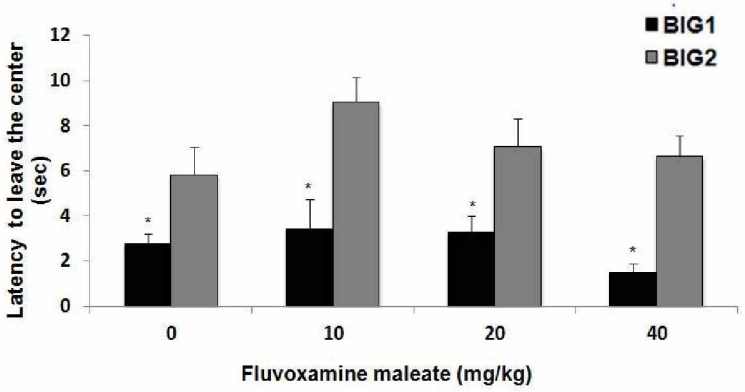
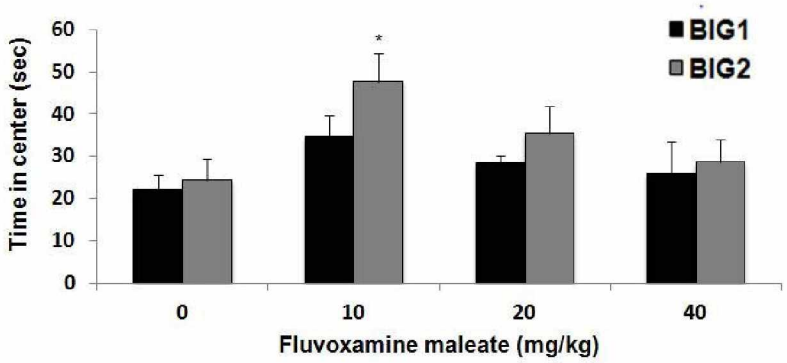


Fig 3.2 Compulsive-like marble burying behavior of the BIG1 and BIG2 strains.

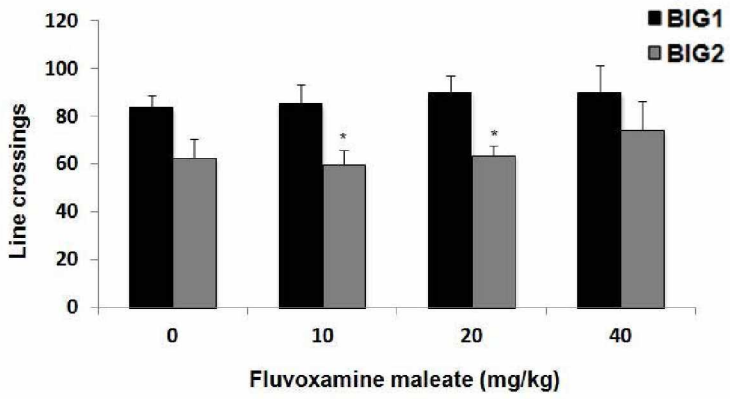
The data represent the mean (± SEM) for total number of marbles buried. ***($p<0.0001$) indicates significant drug effect between 20 and 40 mg/kg with vehicle (0 mg/kg) in both BIG1 and BIG2 strains. 0 mg/kg, n=11 and n=11; 10 mg/kg, n=7 and n=12; 20 mg/kg, n=10 and n=8; 40 mg/kg, n=6 and n=9 for BIG1 and BIG2, respectively.



(a)



(b)



(c)

Fig 3.3 Anxiety-like open field behavior of the BIG1 and BIG2 strains.

Fig 3.3 continued: The data represent the mean (± SEM) for (a) latency to leave the central square, *($p<0.05$) indicates significant strain differences between BIG1 and BIG2 males in each treatment group, (b) the time spent in the central square, *($p<0.05$) indicates significant drug effect between 10 mg/kg with vehicle (0 mg/kg) in BIG2 strain, and (c) the total number of line crossings, *($p<0.05$) indicates strain differences between BIG1 and BIG2 for the 10 and 20 mg/kg treatment groups. 0 mg/kg, n=11 and n=11; 10 mg/kg, n=7 and n=12; 20 mg/kg, n=10 and n=8; 40 mg/kg, n=6 and n=9 for BIG1 and BIG2, respectively.

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Chapter 4: Ovarian Sex Hormones Modulate Compulsive, Affective and Cognitive Functions in A Non-Induced Mouse Model of Obsessive-Compulsive Disorder.[3]

4.1 Abstract

There is currently a lack of understanding of how surgical menopause can influence obsessions, compulsions and associated affective and cognitive functions in female obsessive-compulsive disorder (OCD) patients. Early menopause in women due to surgical removal of ovaries not only causes dramatic hormonal changes, but also may induce affective and cognitive disorders. Here, we tested if surgical removal of ovaries (ovariectomy, OVX), which mimics surgical menopause in humans, would result in exacerbation of compulsive, affective and cognitive behaviors in mice strains that exhibit a spontaneous compulsive-like phenotype. Female mice from compulsive-like BIG, non-compulsive SMALL and randomly-bred Control strains were subjected to OVX or sham-surgery. After 7 days animals were tested for nest building and marble burying to measure compulsive-like behavior. The elevated plus maze and open field tests measured anxiety-like behaviors, while memory was assessed by the novel object recognition. Acute OVX resulted in exacerbation of compulsive-like and anxiety-like behaviors in compulsive-like BIG mice. No significant effects of OVX were observed for the non-compulsive SMALL and Control strains. Object recognition memory was impaired in compulsive-like BIG female mice compared to the Control mice, without an effect of OVX on the BIG mice. We also tested whether 17 β-estradiol (E2) or progesterone (P4) could reverse the effects of OVX. E2, but not P4, attenuated the compulsive-like behaviors in compulsive-like BIG OVX female mice. The actions of the sex steroids on anxiety-like behaviors in OVX females were strain and behavioral test dependent. Altogether, our results indicate that already existing

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compulsions can be worsened during acute ovarian deprivation concomitant with exacerbation of affective behaviors and responses to hormonal intervention in OVX female mice can be influenced by genetic background.

4.2 Introduction

Obsessive-compulsive disorder (OCD) is characterized by intrusive thoughts (obsessions) and/or repetitive behaviors (compulsive rituals) in response to the obsessions (American Psychiatric Association, 2013). OCD has a lifetime prevalence of around 2.3% in the United States (Ruscio et al., 2010) and it has been listed as a common mental disorder in adults (Eaton et al., 2008). The obsessive beliefs lead to compulsive symptoms among patients. For example, contamination obsessions can result in compulsive cleaning (Wheaton et al., 2010). Moreover, OCD can negatively impact cognitive and affective functions in humans. Human studies involving neurocognitive tests and image analysis showed impairments in non-verbal (Kashyap et al., 2013), spatial working (Van der Wee et al., 2007; Nakao et al., 2009) and visual memories (Dirson et al., 1995). Associated comorbidities like depression (Peris et al., 2010; Remijnse et al., 2013) and anxiety disorders (Nestadt et al., 2001) are also very common in the OCD condition.

Clinical and genetic data for OCD corroborate the hypothesis of sexual dimorphism, which reveals differences in clinical manifestations between males and females (Labad et al., 2008; Torresan et al., 2009). Obsessions for cleaning and compulsive contamination are more prevalent in females than males, while males have higher rates of symmetrical and sexual obsessions when compared to females (Noshirvani et al., 1991; Lensi et al., 1996; Bogetto et al., 1999; de Mathis et al., 2008; Labad et al., 2008). There is also a sex difference to treatment response (Mundo et al., 1999). Women typically have a later onset when compared to men and display a bi-modal distribution with the first peak occurring between 13–16 years of age and the second peak around 22–32 years. These are puberty and child bearing stages in a women's life, respectively (Brandes et al., 2004) when sex hormone (estrogen and progesterone, P4) levels are known to fluctuate.

It is well established that a plausible cause of OCD is abnormal cortical-striatal-thalamic circuitry activation (Ahmari et al., 2013) and altered serotonergic (Schilman et al., 2010), glutamatergic (Arnold et al., 2004; Egashira et al., 2013; Porton et al., 2013) and GABAergic (Egashira et al., 2013) systems. Interestingly, female hormones, such as estrogen and P4, regulate various neurotransmitter signaling pathways in brain regions implicated in OCD (Dreher et al., 2007; Karakaya et al., 2007; Benmansour et al., 2009; Alonso et al., 2011; Quinlan et al., 2013; Barth et al., 2015). During the estrous phase, circulating estrogen levels are higher and serotonin release is lower in striatal neurons (Yang et al., 2015), while in the frontal cortex, estrogen depletion by ovariectomy (OVX) decreases 5-HT2A receptor density and mRNA levels (Cyr et al., 1998). On the other hand, P4 increases dopamine release mediated by NMDA receptor activation in striatal neurons (Cabrera and Bregonzio, 1996) and decreases NMDA binding density in the frontal cortex after OVX (Cyr et al., 2000). Therefore, ovarian sex hormones may account for the sex differences observed in OCD.

Women are subjected to hormonal fluctuations during their entire life span, which may lead to significant alterations in mood and cognition (Soares and Zitek, 2008). However, remarkable challenges are encountered during the menopause transition due to the natural decline in ovarian function, the primary source of estrogen and P4 (Luine, 2014). Natural menopause is also associated with cognitive deficits and mood disorders (Weber et al., 2012; Dumas et al., 2013). Such dysfunction in mood and cognitive functions has also been reported in women with surgical menopause (Chen et al., 2013; Faubion et al., 2015). Physiological challenges during surgical menopause are much more drastic due to a sudden depletion of ovarian sex steroids as compared to progressive menopause which follows fluctuating patterns of steroid levels (Bachmann, 2001; Rodriguez and Shoupe, 2015; Rodríguez-Landa et al., 2015). This results in greater predisposition to mood and anxiety disorders when compared to natural menopause (Rodríguez-Landa et al., 2015).

The impact of acute ovarian dysfunction during surgical menopause on compulsive behaviors and comorbid affective behaviors in females are currently poorly understood. In addition to younger women, one out of eight women after the age of 55 undergoes bilateral oophorectomy (surgical removal of ovaries) before reaching natural menopause due to benign diseases, prophylaxis against cancer and autoimmune disorders (Shuster et al., 2010; Erekson et al., 2013; Cox and Liu, 2014). Existing studies have investigated obsessions and compulsions only during and after progressive menopause with contradictory evidence (Vulink et al., 2006). One such study showed that, OCD is not a rare comorbidity during post menopause (Uguz et al., 2010), while another study demonstrated that the symptoms are more related with menarche and decreases during menopause (Guglielmi et al., 2014).

In animal studies, acute administration of estradiol (E2) in pre-pubertal female rats exerted an anti-compulsive-like effect (Flaisher-Grinberg et al., 2009), while male mice with estrogen deficiency (aromatase enzyme knockout) displayed compulsive-like behavior (Hill et al., 2007). In OVX rats, concurrent administration of E2 and P4 was able to reduce compulsive-like lever pressing behavior (Fernández-Guasti et al., 2006). Most of these studies were conducted on induced (drug or gene knockouts) models and did not investigate the associated comorbidities like anxiety and cognitive impairments, while only one study looked at the effect of P4 and E2 in the ovariectomized condition (Fernández-Guasti et al., 2006). Moreover, though OCD has a compelling genetic basis (Nestadt et al., 2010) the role of genetic background in influencing steroid actions in OCD condition during menopause has never been explored.

How do already existing compulsions in females get affected during acute sex hormone deprived conditions when compared to non-compulsive females are not clearly known. The co morbid anxiety and cognitive functions associated with OCD during such a physiological state and the role of genetic background in influencing steroid actions demands investigation. According to Maio et al. (2014), our mice developed through selective breeding for phenotypes

of increased or decreased amounts of compulsive-like behavior can be a heuristic tool for studying OCD, especially the replicate BIG strains (BIG1 and BIG2). An unpublished study from our lab has shown that there is variation in compulsive-like and affective behaviors between the two replicate BIG strains that mimics heterogeneity as seen in subgroups of OCD patients. This study however did not look into hormonal deprivation and manipulations. We therefore investigated the hypothesis that acute deprivation of estrogen and P4 through OVX for 7 days will increase the compulsive-and anxiety-like behavior and impair novel object recognition memory in compulsive-like mouse strains. We also hypothesized that the administration of estrogen (E2) and P4 will attenuate the exacerbation in compulsive-like, anxiety-like and cognitive behaviors in compulsive-like strains. Though rodents do not have menopause, surgical removal of the ovaries can cause depletion of E4 and P4 (Kato et al., 2013). We therefore used bilateral OVX as the sex hormone deprived surgical menopause model to achieve the experimental endpoints in this study.

4.3 Materials and Methods

The University of Alaska Fairbanks Institutional Animal Care and Use Committee approved the animal care and experimental procedures (IACUC assurance numbers 568518 and 631126).

4.3.1 Mouse Husbandry

All mice were raised in polypropylene cages (27 cm × 17 cm × 12 cm) and provided with wood shavings under a 12:12 light-dark cycle at 22 ± 1°C. Weaning of the pups was conducted at 19–21 days of age. All mice were housed with same-sex and same-strain littermates until the end of all the experiments. All mice were singly housed just before the behavioral assessments and were returned to their home cages with their littermates following each test. Food (Masuri Rodent Diet #5663, Purina Mills, LLC, St. Louis, MO, USA) and water were available ad libitum.

4.3.2 Experimental Subjects

The mouse model of OCD used for this study was developed from house mouse (Mus musculus) strains bidirectionally selected for nest-building behavior (Lynch, 1980; Bult and Lynch, 2000). The stock population for the original selection experiment (Lynch, 1980) was a cross among eight inbred strains, i.e., A, AKR, BLB/c, C3H/2, C57BL, DBA/2, Is/Bi, and RIII, to yield the HS/Ibg outbred strain (McClearn et al., 1970; Lynch, 1980). This resulted in two BIG strains (BIG1 and BIG2) that use a forty-fold larger amount of cotton for their nest than the two SMALL strains (SML1 and SML2) and two randomly-bred control strains (C1 and C2) that show intermediate levels of nesting (Lynch, 1980; Bult and Lynch, 2000). The BIG strains engage in excessive and repetitive nest building (considered to be homologous to hoarding in humans; Warneke, 1993) and marble burying behavior which is dose-dependently attenuated by fluoxetine and clomipramine, but not desipramine, treatment, making the BIG mice a novel non-induced model for OCD (Greene-Schloesser et al., 2011).

For the OVX study, female mice (Mus musculus) of six different mouse strains i.e., two each of compulsive-like strains (BIG1 and BIG2), randomly-bred Control strains (C1, C2) and SMALL (SML1, SML2) strains, were used. For the hormone replacement studies in OVX females, only compulsive-like BIG1 and BIG2 female strains were used. All mice were 80–90 days of age during testing. All data were collected by an individual blinded to the outcome of the study.

4.3.3 Surgical Procedure

For the OVX study, animals were divided into two groups for each strain. One group was sham operated while the other group was OVX (removal of ovaries). All animals in the hormone replacement studies were OVX. For the surgical procedures, females were exposed to isofluroane (4% induction and flow rate of 1.5–2 L/min) anesthesia. Abdominal incisions were made longitudinally and bilaterally in the region below the last lumbar vertebra. The ovary,

oviduct and top of the fallopian tubes were tied and removed in the OVX group. For the sham-operated mice, the procedure remained the same except that the ovaries were not removed but only identified (Fonseca et al., 2013). The abdominal wall and the skin were sutured as described by Capettini et al. (2011). All animals were provided ibuprofen in the drinking water 24 h prior to surgery and maintained for 3–4 days post surgery as needed for pain management.

4.3.4 Hormone Administration

4.3.4.1 E2 Administration

For the E2 administration study, BIG1 and BIG2 females were subdivided into two treatment groups: vehicle and E2 (n = 12 females per group). Seven days after OVX, the vehicle group received a single subcutaneous injection of corn oil while the E2 group received 0.1 mg/kg (acute dosage of E2 produces comparable proestrus levels (Walf et al., 2006)) of E2 (Sigma, St. Louis, MO, USA) in corn oil 44 h before behavioral assessments (compulsive and anxiety tasks; Walf et al., 2008b). For the object recognition task, the mice were injected immediately after the training session and were tested 4 h later (Walf et al., 2008a). A total gap of 5 days between each behavior was employed.

4.3.4.2 P4Administration

For the P4 administration study, BIG1 and BIG2 females were subdivided into vehicle and P4 groups (n = 9 females per group). Following 7 days of OVX, the vehicle group received corn oil while the P4 group received 4 mg/kg of P4 1 h before behavioral testing. For the object recognition task, the mice were injected immediately after the training session and the test was performed 4 h later (Walf et al., 2008a). A gap of 3 days between the end of each behavioral test and the next injection was employed. An acute dosage of P4 used in this study approximates circulating and central P4 levels observed during the proestrus phase (Walf et al., 2006).

4.3.5 Plasma Steroid Levels

To establish that acute OVX leads to depletion of E2 and P4 plasma E2 and P4 levels were determined in plasma samples (n = 5–7 per group) of OVX and sham operated compulsive-like BIG female strains (BIG1 and BIG2). All samples were assayed in duplicates using Cayman ELISA kits (Ann Arbor, MI, USA) as per the manufacturer's instructions. Data collection was accomplished with a Biotek EL808 spectrophotometric plate reader and analyzed by Prism software.

4.3.6 Compulsive-Like Behaviors

4.3.6.1 Nest-Building

Nest-building behavior was used to assess the compulsive-like phenotype of the female mice (Greene-Schloesser et al., 2011). All mice were housed individually and were allowed to access a pre-weighed cotton roll placed in the cage top food hopper. The amount of cotton used by the mice after 24 h was determined by weighing the cotton roll. As all other behavioral assessments in the P4 administration experiment were performed after 1 h of P4 administration, nest building was measured for 1 h and 24 h of cotton availability, starting 1 h after the injection, to be able to capture the short-term effects of P4 and also to be able to compare this behavior to the 24-h nesting score of the E2 administration experiment.

4.3.6.2 Marble Burying

The marble-burying test was also used to measure compulsive-like behavior (Takeuchi et al., 2002; Thomas et al., 2009; Greene-Schloesser et al., 2011; Angoa-Pérez et al., 2013). All mice were individually introduced to a polypropylene cage (37 cm × 21 cm × 14 cm) containing 20 glass marbles (10 mm in diameter) evenly spaced on 5 cm deep wood shavings firmly pressed into a bedding without access to food or water for 20 min. The total number of marbles buried at least 2/3 in the 20-min period was quantified as compulsive-like digging behavior

(Greene-Schloesser et al., 2011). After the 20-min test, the animals were returned to their home cages with littermates.

4.3.7 Anxiety-Like Behaviors

4.3.7.1 Open Field

The open field test was performed to evaluate anxiety-like behavior in female mice (Crawley, 1985; Meerlo et al., 1999). Female mice were singly housed outside the testing room just prior to testing. The open field apparatus consisted of an open field arena (40 cm × 40 cm × 30 cm). For testing, animals were placed in the center of the field and allowed to explore the arena for 3 min. Entries into the central square (20 cm × 20 cm) (Greene-Schloesser et al., 2011) were recorded by ANYMaze video tracking system (Stoelting Co., Wood Dale, IL, USA). Total number of line crossings was also assessed for sham and OVX strains. The apparatus was cleaned before each test.

4.3.7.2 Elevated Plus Maze

Anxiety-like behavior was further substantiated by the elevated plus maze test. The plus maze apparatus consisted of two open arms (5 cm × 40 cm) and two closed arms (5 cm × 40 cm × 20 cm) at right angles to each other. Each mouse was placed in the central square facing an open arm and was allowed to explore the maze for 5-min duration (Frye et al., 2008). The time spent on the open arms was determined by the ANYMaze video tracking program (Stoelting Co., Wood Dale, IL, USA). The maze was cleaned before each test.

4.3.8 Novel Object Recognition Test

The novel object recognition test was performed to measure object recognition memory (Antunes and Biala, 2012). Mice were allowed to explore the open field arena (40 cm × 40 cm × 30 cm) without any objects for 3 min during the habituation phase on day 1. Twenty-four hours later on day 2, the training session was performed and mice were introduced to two similar

objects (plastic toys) within a 5 cm distance in the open field arena for 3 min. Mice were then taken out of the arena and returned to their home cages. After 4 h, one of the objects was replaced with a novel object of different shape and size. Animals were then reintroduced into the arena and allowed to explore the objects for 3 min in the testing phase. Time spent exploring the familiar and novel objects was recorded with ANYMaze video tracking software (Stoelting Co., Wood Dale, IL, USA). The preference of one object over another was assessed through the Recognition Index (RI: time spent on novel object divided by the time spent on novel and familiar object together; Fonseca et al., 2013).

4.3.9 Statistical Analysis

All data were analyzed using Statistical Analysis System (SAS) software. A general linear model (GLM) repeated analysis of variance (ANOVA), with strain (BIG, SMALL, Control), replicate nested within strain (1, 2), treatment (OVX, sham), and strain by treatment interaction effects was used to statistically evaluate the effects of OVX on nest building behavior (grams of cotton), marble burying behavior (number of marbles buried), open field behavior (time in seconds in center), elevated plus maze behavior (time in seconds on open arms), and novel object recognition memory (RI). If the replicate nested within strain effect was significant, the strain effect was tested over the replicate effect. If the replicate effect was not significant, the strain effect was tested over the error term.

A GLM ANOVA, with treatment (OVX, sham), strain (BIG1, BIG2), and replicate by treatment interaction effects, was used to statistically evaluate the effects of OVX on E2 (pg/mL) and P4 (ng/mL) plasma levels.

A GLM ANOVA, with treatment (E2, vehicle or P4, vehicle), strain (BIG1, BIG2), and strain by treatment interaction effects, was used to statistically evaluate the effects of females, sex hormone replacement in compulsive-like OVX females on nest building behavior (1 and 24

h nesting scores), marble burying behavior, open field behavior, elevated plus maze behavior and novel object recognition memory.

When significance was found appropriate pairwise comparisons were performed using the studentized range test. The nesting scores were square root transformed to obtain a more normal distribution (Bult and Lynch, 1996, 1997, 2000), while the data are presented as non-transformed nesting scores.

4.4 Results

4.4.1 Acute OVX Increased Compulsive-Like Behavior in Compulsive-Like BIG Strains

4.4.1.1 Compulsive-Like Nesting

Acute OVX in compulsive-like BIG1 (post hoc $t_{(22)} = 8.983$, $p < 0.0001$) and BIG2 (post hoc $t_{(22)} = 11.51$, $p < 0.0001$) females resulted in significant increases of nesting behavior when compared to the sham operated ones ($F_{(1,134)} = 77.60$, $p < 0.0001$). No significant increases of compulsive-like nesting were observed in the SMALL (SML1: post hoc $t_{(21)} = 0.0045$, $p > 0.99$; SML2: post hoc $t_{(22)} = 0.794$, $p > 0.43$) and Control (C1: post hoc $t_{(22)} = 0.270$, $p > 0.78$; C2: post hoc $t_{(22)} = 0.0150$, $p > 0.98$) OVX strains when compared to their sham operated controls (Figure 4.1A), which explains the significant strain by treatment interaction effect ($F_{(2,134)} = 65.91$, $p < 0.0001$). The BIG strains built bigger nests than the SMALL and Control mice ($F_{(2,3)} = 70.84$, $p < 0.0001$). The replicate nested within strain effect was also significant ($F_{(3,134)} = 10.59$, $p < 0.0001$), predominantly due to the BIG1 females building bigger nests than the BIG2 females (sham: post hoc $t_{(22)} = 5.188$, $p < 0.0001$; OVX: post hoc $t_{(22)} = 2.666$, $p < 0.05$).

4.4.1.2 Compulsive-Like Marble Burying

Acute OVX resulted in more marbles buried in BIG1 (post hoc $t_{(22)} = 3.248$, $p < 0.004$) and BIG2 (post hoc $t_{(22)} = 3.193$, $p < 0.005$) females when compared to the sham operated groups ($F_{(1,134)} = 18.15$, $p < 0.0001$). No significant differences were observed between OVX and

sham operated SMALL (SML1: post hoc $t_{(21)}$ = 0.525, p > 0.60; SML2: post hoc $t_{(22)}$ = 1.028, p > 0.31) and Control (C1: post hoc $t_{(22)}$ = 1.732, p > 0.09; C2: post hoc $t_{(22)}$ = 0.650, p > 0.52) strains (Figure 4.1B), which explains the significant strain by treatment interaction effect ($F_{(2,134)}$ = 3.49; p < 0.034). BIG females buried more marbles than the SMALL females, with the Control mice showing intermediate values ($F_{(2,3)}$ = 24.24, p < 0.015). The replicate nested within strain effect was also significant ($F_{(3,134)}$ = 5.56, p < 0.0013), predominantly due to the SML1 females burying fewer marbles than the SML2 females (sham: post hoc $t_{(21)=}$ 2.324, p < 0.05; OVX: post hoc $t_{(22)}$ = 2.922, p < 0.008).

4.4.2 Acute OVX Increased Anxiety-Like Behavior in Compulsive-Like BIG Strains

4.4.2.1 Anxiety-Like Open Field Behavior

In the anxiety-like open field test, the BIG1 (post hoc $t_{(22)}$ = 5.697, p < 0.0001), BIG2 (post hoc $t_{(22)}$ = 5.008, p < 0.0001), C1 (post hoc $t_{(22)}$ = 6.272, p < 0.0001), C2 (post hoc $t_{(22)}$ = 5.927, p < 0.0001), and SML1 (post hoc $t_{(21)}$ = 2.296, p < 0.032) OVX females spent significantly less time in the center when compared to the sham groups ($F_{(1,134)}$ = 119.24, p < 0.0001). No significant difference was observed between OVX and sham groups in SML2 (post hoc $t_{(22)}$ = 1.286, p > 0.21) females for the time spent in the center (Figure 4.2A), which explains the significant strain by treatment interaction effect ($F_{(2,134)}$ = 10.55; p < 0.0015). No significant strain effect was found ($F_{(2,3)}$ = 6.51, p > 0.08), although the Control strains tended to be the least anxious and the SMALL strains the most, while the BIG strains tended to be intermediate. The replicate nested within strain effect was significant ($F_{(3,134)}$ = 22.87, p < 0.0001), predominantly due to the C1 females spending less time in the center than the C2 females (sham: post hoc $t_{(22)}$ = 5.582, p < 0.0001; OVX: post hoc $t_{(22)}$ = 5.927, p < 0.0001). For total number of line crossings, as a measure of locomotor activity (Figure 4.2B), no differences were observed among the strains ($F_{(2,3)}$ = 0.10, p > 0.90) and between sham and OVX groups ($F_{(1,134)}$ = 0.03, p > 0.80).

4.4.2.2 Anxiety-Like Elevated Plus Maze Behavior

Acute OVX resulted in less time spent on the open arms in the elevated plus maze test for BIG1 ($t_{(22)}$ = 6.320, $p < 0.0001$) and BIG2 ($t_{(22)}$ = 4.934, $p < 0.0001$) females when compared to the sham groups ($F_{(1,134)}$ = 30.14, $p < 0.0001$). No significant differences were observed in Control (C1: post hoc $t_{(22)}$ = 0.0676, $p > 0.94$ and C2: post hoc $t_{(22)}$ = 1.403, $p > 0.17$) and SMALL (SML1: post hoc $t_{(21)}$ = 0.1833, $p > 0.85$ and SML2: post hoc $t_{(22)}$ = 0.4394, $p > 0.66$) OVX strains when compared to the sham operated mice (Figure 4.2C), which explains the significant strain by treatment interaction effect ($F_{(2,134)}$ = 17.50, $p < 0.0001$). The BIG females spent the most time on the open arms, followed by the Control females, and the SMALL mice showed the highest level of anxiety-like behavior ($F_{(2,134)}$ = 26.84; $p < 0.0001$). The replicate nested within strain effect was not significant ($F_{(3,134)}$ = 0.86, $p > 0.46$).

4.4.3 Acute OVX Did Not Affect Recognition Index (RI) for Compulsive-Like BIG Strains in Novel Object Recognition

The RI was significantly reduced in OVX females compared to sham operated mice ($F_{(1,134)}$ = 3.94; $p < 0.05$; Figure 4.3) with the C2 OVX females having a significantly lower RI than the sham operated C2 mice (post hoc $t_{(22)}$ = 2.569, $p < 0.05$), while the other strains did not show significant differences (BIG1: post hoc $t_{(22)}$ = 0.02763, $p > 0.97$; BIG2: post hoc $t_{(22)}$ = 0.2579, $p > 0.78$; C1: post hoc $t_{(22)}$ = 0.4236, $p > 0.66$; SML1: post hoc $t_{(21)}$ = 0.6280, $p > 0.52$; SML2: post hoc $t_{(22)}$ = 0.9393, $p > 0.34$). The Control females had significantly higher RIs than the BIG and SMALL mice ($F_{(2,134)}$ = 37.70; $p < 0.0001$). The replicate nested within strain ($F_{(3,134)}$ = 0.53, $p > 0.66$) and the strain by treatment interaction ($F_{(2,134)}$ = 0.92, $p > 0.40$) effects were not significant.

4.4.4 Plasma E2 and P4 Levels Declined in BIG Strains Following Acute OVX

Plasma E2 levels in acute OVX BIG1 (post hoc $t_{(9)}$ = 5.501 $p < 0.0001$) and BIG2 (post hoc $t_{(10)}$ = 6.948 $p < 0.0001$) mice were significantly and similarly (strain: $F_{(1,19)}$ = 0.00; $p > 0.99$;

strain by treatment interaction: $F_{(1,19)} = 2.99$; $p > 0.10$) reduced when compared to the sham females (treatment: $F_{(1,19)} = 63.23$; $p < 0.0001$; Figure 4.4A). P4 levels were significantly reduced in the BIG2 (post hoc $t_{(12)} = 8.665$ $p < 0.0001$) but not in BIG1 (post hoc $t_{(10)} = 1.993$ $p > 0.058$) OVX females when compared to their sham counterparts ($F_{(1,19)} = 61.17$; $p < 0.0001$; Figure 4.4B), which explains the significant strain ($F_{(1,19)} = 17.30$; $p < 0.0005$) and strain by treatment interaction ($F_{(1,19)} = 18.93$; $p < 0.0004$) effects.

4.4.5 Acute E2 and P4 Administration in OVX Female Mice

4.4.5.1 Compulsive-Like Nesting Was Attenuated by E2 But Not P4 Administration

Acute E2 administration resulted in a significant and similar (strain by treatment interaction: $F_{(1,43)} = 0.00$, $p > 0.94$) decline of nesting scores in the BIG1 (post hoc $t_{(22)} = 3.000$, $p < 0.007$) and BIG2 (post hoc $t_{(22)} = 3.814$, $p < 0.001$) OVX females when compared to the vehicle controls ($F_{(1,43)} = 19.51$; $p < 0.0001$; Figure 4.5A). The BIG1 females used more cotton for their nest compared to the BIG2 mice ($F_{(1,43)} = 5.69$, $p < 0.022$), which replicated the results in Figure 4.1A. No significant differences were observed in the 1 h ($F_{(1,32)} = 0.96$; $p > 0.33$) and 24 h ($F_{(1,32)} = 3.47$; $p > 0.05$) nesting scores of acute P4 treated BIG1 and BIG2 OVX females when compared to the vehicle treated controls (Figures 4.6A,B). For 1 h and 24 h nesting scores, the strain ($F_{(1,32)} = 0.37$; $p > 0.54$; $F_{(1,32)} = 1.05$; $p > 0.31$, respectively) and strain by treatment interaction ($F_{(1,32)} = 2.06$; $p > 0.16$; $F_{(1,32)} = 0.00$; $p > 0.95$, respectively) effects were not significant.

4.4.5.2 Compulsive-Like Marble Burying was Attenuated by E2 But Not P4 Administration

BIG1 (post hoc $t_{(22)} = 6.447$, $p < 0.0001$) and BIG2 (post hoc $t_{(22)} = 7.606$, $p < 0.0001$) OVX females buried significantly less marbles in the acute E2 treatment group when compared to the vehicle groups ($F_{(1,43)} = 85.67$, $p < 0.0001$; Figure 4.5B). The strain ($F_{(1,43)} = 0.79$, $p > 0.37$) and strain by treatment interaction ($F_{(1,43)} = 1.24$, $p > 0.27$) effects were not significant,

indicating that the BIG1 and BIG2 OVX females had similar marble burying scores and responses to E2.

P4 administration did not cause significant changes in the number of marbles buried by BIG1 and BIG2 OVX females when compared to the vehicle control mice ($F_{(1,32)} = 2.64$; $p > 0.11$; Figure 4.6C). The strain ($F_{(1,32)} = 3.22$, $p > 0.08$) and strain by treatment interaction ($F_{(1,32)} = 1.64$, $p > 0.20$) effects were also not significant.

4.4.5.3 E2 and P4 Treatment Showed Strain Dependent Decreases in Anxiety-Like Behavior in the Open Field

In the acute E2 administration group, the BIG1 OVX females (post hoc $t_{(22)} = 4.245$, $p < 0.0005$) spent more time in the center when compared to the vehicle group ($F_{(1,43)} = 16.70$, $p < 0.0002$; Figure 4.5C). No significant difference in the time spent in the center was observed between E2 and vehicle groups in BIG2 OVX females (post hoc $t_{(22)} = 1.564$ $p > 0.13$). The significant strain effect ($F_{(1,43)} = 17.05$; $p < 0.0003$) was due to BIG1 E2 administered females performing better than the BIG2 E2 administered females (post hoc $t_{(22)} = 4.178$, $p < 0.0005$). The strain by treatment interaction effect was not significant ($F_{(1,43)} = 3.77$, $P > 0.058$), which showed that the BIG2 females responded to E2 in a similar direction as the BIG1 females.

P4 administered groups in both BIG1 (post hoc $t_{(16)} = 4.311$, $p < 0.0005$) and BIG2 (post hoc $t_{(16)} = 3.904$, $p < 0.001$) OVX females spent significantly more time in the center when compared to their respective vehicle control groups ($F_{(1,32)} = 33.75$; $p < 0.0001$; Figure 4.6D). The strain ($F_{(1,32)} = 2.17$, $p > 0.15$) and strain by treatment interaction ($F_{(1,32)} = 0.08$, $p > 0.77$) effects were not significant, indicating that the BIG1 and BIG2 OVX females spent similar times in the center and responded similarly to P4.

4.4.5.4 P4, But Not E2, Treatment Had an Effect on Anxiety-Like Elevated Plus Maze Behavior

No significant differences in the time spent on the open arm in the elevated plus maze test was observed between E2 and vehicle groups of BIG1 and BIG2 OVX females ($F_{(1,43)}$ = 1.81; $p > 0.18$; Figure 4.5D). In addition, the strain ($F_{(1,43)}$ = 0.22, $p > 0.63$) and strain by treatment interaction ($F_{(1,43)}$ = 0.39, $p > 0.53$) effects were also not significant.

Overall, P4 administration significantly increased the time spent on the open arms compared to the vehicle groups ($F_{(1,32)}$ = 8.20, $p < 0.0073$; Figure 4.6E), which was significant when comparing the BIG2 P4 treated to the BIG2 vehicle OVX females (post hoc $t_{(16)}$ = 2.692, $p < 0.014$). Although the trend was in the same direction as the BIG2 OVX females, no significant difference was observed between P4 and vehicle groups of BIG1 OVX females (post hoc $t_{(16)}$ = 1.358, $p > 0.18$). The strain ($F_{(1,32)}$ = 0.63, $p > 0.43$) and strain by treatment interaction ($F_{(1,32)}$ = 0.89, $p > 0.35$) effects were not significant, indicating that the BIG1 and BIG2 OVX females spent similar times on the open arms, and the BIG1 females responded to P4 in a similar direction as the BIG2 females.

4.4.5.5 E2 and P4 Improved Recognition Index (RI) in Object Recognition Memory With a Replicate Effect Seen in E2 Treatment

Overall, E2 administration significantly increased performance in the novel object recognition test compared to the vehicle treated OVX females ($F_{(1,43)}$ = 26.95, $p < 0.0001$; Figure 4.5E), which was significant for the BIG2 OVX females compared to their vehicle treated counterparts (post hoc $t_{(22)}$ = 5.358, $p < 0.0001$). Although the trend was in the same direction as the BIG2 OVX females, no significant difference was observed in the RI of E2 treated BIG1 OVX females compared to vehicle treated females (post hoc $t_{(22)}$ = 1.946, $p > 0.06$), which explains the significant strain by treatment interaction effect ($F_{(1,43)}$ = 5.55, $p < 0.024$). The strain effect was not significant ($F_{(1,43)}$ = 1.93, $p > 0.17$), which indicates that the BIG1 and BIG2 females had overall similar memory scores.

P4 administration enhanced the performance in the novel object recognition test for both the BIG1 (post hoc $t_{(16)} = 3.855$, $p < 0.001$) and the BIG2 (post hoc $t_{(16)} = 4.726$, $p < 0.0001$) OVX females compared to their vehicle control groups ($F_{(1,32)} = 114.3$; $p < 0.0001$; Figure 4.6F). The BIG1 OVX females had higher RIs compared to the BIG2 OVX females (strain: $F_{(1,32)} = 7.32$; $p < 0.011$, irrespective of treatment group (strain by treatment interaction: $F_{(1,32)} = 0.36$; $p > 0.55$)).

4.5 Discussion

In the current study we showed that acute OVX for 7 days resulted in a significant increase in the compulsive-like nesting and marble burying behaviors of BIG1 and BIG2 female mice. No increase in nesting and marble burying was observed for the Control and SMALL strains, which shows the specificity of the OVX effects for the compulsive-like condition. The exacerbations in compulsive-like behaviors in BIG mice were attenuated by acute subcutaneous administration of E2, but not P4. Human studies have shown that gonadal steroids trigger or precipitate mood disorders in women with a history of an already existing disease condition when compared to women without it (Hay et al., 1994; Schmidt et al., 1998; Clayton and Ninan, 2010). Onset and exacerbation of OCD associated with pregnancy and postpartum has been shown in human studies (Neziroglu et al., 1992; Williams and Koran, 1997; Labad et al., 2005; Uguz et al., 2007; Forray et al., 2010) establishing a strong link between reproductive events and OCD. However, there is lack of literature on how obsessions and compulsions and associated affective and cognitive behaviors vary during induced menopause. According to a review (Forray et al., 2010) a large variation exists in human studies on onset and exacerbation of OCD during reproductive events and one of the contributing factors could be innate differences in patient populations. In congruence with this we found that BIG1 sham females had higher nesting scores when compared to BIG2 sham females. This variation in compulsive-like nesting behavior was also seen post acute OVX but was abolished in the E2 treatment

regimen. What is more intriguing is the fact that BIG1 and BIG2 females did not exhibit variation in compulsive-like marble burying. This is an interesting finding indicating heterogeneity in the BIG strains based on compulsive-like traits and genetic background as often seen in subgroups of OCD patients (Fontenelle et al., 2005; Grados and Riddle, 2008; Leckman et al., 2009). Whether E2 might be more effective compared to P4 in reducing OCD symptoms in postmenopausal females, as seen in our OVX compulsive-like mice, remains to be elucidated.

A prior study has shown that acute E2 administration along with P4 to OVX rats reduced compulsive perseverance in the T-maze (Fernández-Guasti et al., 2006). This is similar to our findings in the compulsive-like mice though, E2 alone had an attenuating effect in the OVX state. Interestingly, previous findings show that acute P4 administration reduced compulsive-like marble burying behavior in male rats (Umathe et al., 2009). However, in the current study we did not see an anti-compulsive effect of P4 treatment in BIG mice. This could be due to various factors, including using mouse strains and females in our study compared to male rats in the (Umathe et al., 2009) study.

In the anxiety-like measures, the OVX BIG strains spent less time in the center of the open field and also explored the open arm less in elevated plus maze when compared to the sham groups. The SMALL and the Control OVX strains showed no significant changes in open field and elevated plus maze when compared to their sham counterparts. Therefore, OVX worsened anxiety-like behaviors in compulsive-like condition specifically. Acute administration of E2 resulted in increased time spent in the central square of the open field in BIG1, but not BIG2 strains, indicating a strain dependent effect in the E2 treatment response. The acute dosage and the time frame of administration of E2 in our study previously also showed anxiolytic effects in the open field in acutely OVX mice (Walf et al., 2008b) and rats (Walf and Frye, 2009). In the plus maze no significant effect of E2 was observed in the compulsive-like mice, which could be

due to the fact that the open field and the elevated plus maze tests measure different aspects of emotionality associated with anxiety (Ramos, 2008; Anchan et al., 2014).

P4 treatment, on the other hand, decreased anxiety-like behavior in open field for both BIG1 and BIG2 strains. For the elevated plus maze, however, P4 was effective only in the BIG2 and not the BIG1 strain. These results indicate a strain specific response to anxiety-like behavior due to P4 administration. Strain specific effects of E2 and P4 on behavioral responses have been sparsely explored in rodent studies. Only one study has shown significant strain specific effects of E2 on depressive-like forced swim behavior (Koss et al., 2012). Behavioral responses to alterations in gonadal steroids have been found to vary in women with and without premenstrual syndrome (Schmidt et al., 1998), which might be similar to the differences in behavioral responses to E2 and P4 in the BIG1 and BIG2 strains.

The association of memory impairment with OCD is not clear. Many clinical studies have failed to find any evidence that OCD is associated with memory deficits (McDonald, 1991; Dirson et al., 1995; Radomsky and Rachman, 1999). In addition, no impairment in declarative and short-term memory has been found in OCD patients compared to normal controls (Roth et al., 2004; Demeter et al., 2013). However, many others have reported working memory impairments in patients (Martin et al., 1995; Nakao et al., 2009). Our BIG mice showed a larger object recognition memory deficit than the Control mice, both in the sham and OVX groups. However, the SMALL mice also showed a similar memory deficit and, therefore, whether this memory deficit in the BIG mice was due to a genetic correlation between compulsive-like behaviors and object recognition memory, or was due to genetic differences between the two strains caused by founder effects or random drift (Bult and Lynch, 1996, 2000), remains to be elucidated. Also as the BIG and SMALL strains had a RI below 0.5 they appeared to avoid the new object, while the control mice had an index above 0.5 and appeared to favor the new object.

Acute OVX did not result in significant object recognition memory impairment in the compulsive-like condition. Contradictory evidence exists as to whether acute OVX leads to object recognition memory impairment in normal mice (c57 strain). While few studies have shown memory loss during acute OVX (Gresack and Frick, 2006; Rhodes and Frye, 2006), others show the opposite (Willard et al., 2011; Fonseca et al., 2013; Bastos et al., 2015). However, chronic OVX has consistently caused poor performance in the novel object recognition test (Fonseca et al., 2013; Bastos et al., 2015). Though there was no overall effect in novel object recognition memory among sham and OVX strains, E2 treatment improved the RI in only the BIG2 but not the BIG1 strain. P4 treatment however improved the RI in both the strains. In the current context of investigation object recognition was performed to evaluate short term or working memory impairments associated with OCD in the mouse strains. However, object recognition encompasses just one aspect of assessing otherwise very complex memory consolidation and cognition process in rodents. In future studies we aim to include a more robust assessment of both short-term and spatial memory components.

The current study supports a complex interplay of genetic background and sex steroids during acute ovarian dysfunction in the compulsive-like condition. We report exacerbation of compulsive-like behaviors with trait specific intra-strain variation during acute ovarian failure in the spontaneously compulsive-like mouse strains only, which was restored by E2 and not P4. This effect was similar for the spontaneously compulsive-like BIG1 and BIG2 strains, unlike the associated anxiety-like and cognitive-like behaviors, which displayed differences between the BIG1 and BIG2 strains for both E2 and P4 effects on these behaviors. We therefore hypothesize that the associated comorbidities in the surgical menopause state in the OCD condition might vary among individuals because of genetic differences. Future studies will focus on investigating effects of E2 and P4 on the chronic OVX state and also the potential signaling pathways in the brain of compulsive-like mice. Overall, the results presented here strengthen the face, predictive

and construct validities of the mouse model for investigating heterogeneity associated with OCD during ovarian failures in females.

4.6 Acknowledgements

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4.7 Figures

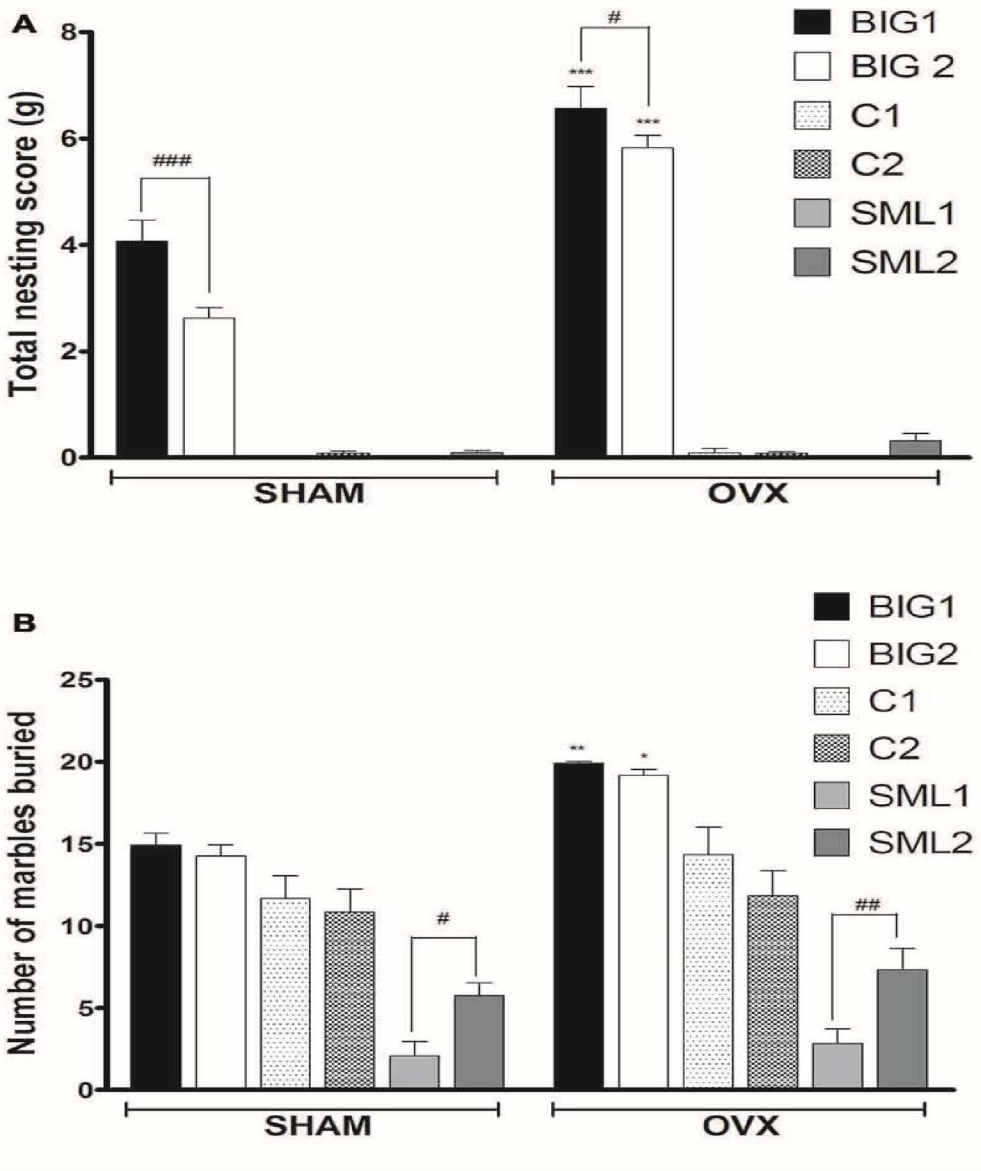

Fig 4.1 Compulsive-like behavior in BIG, SMALL and Control strains.

The data represent the mean (± SEM) for (A) nesting score in grams between 0–24 h in nest-building test and (B) number of marbles buried in marble burying test of the two replicates of the BIG, SMALL and Control strains. *($p < 0.05$), **($p < 0.001$) and ***($p < 0.0001$) indicates significant differences between sham and ovariectomy (OVX) groups. #($p < 0.05$), ##($p < 0.001$) and ###($p < 0.0001$) indicate significant differences between replicate strains.

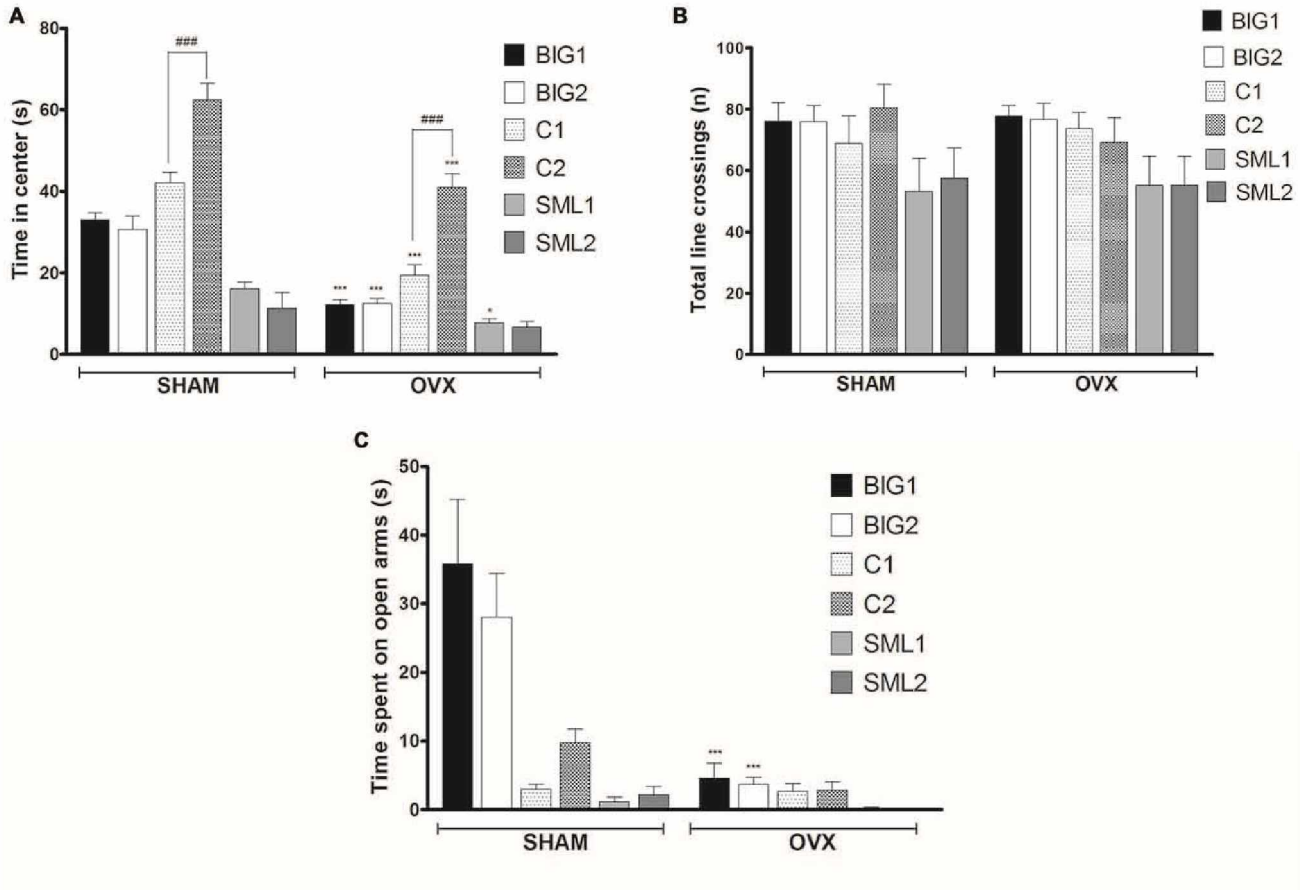

Fig 4.2 Anxiety-like behavior in BIG, SMALL and Control strains.

The data represent the mean (± SEM) for (A) time spent on center in open field and (B) total number of line crossings and (C) time spent on open arm in elevated plus maze of the two replicates of the BIG, SMALL and Control strains. ***($p < 0.0001$) and *($p < 0.05$) indicates significant differences between sham and OVX groups. ###($p < 0.0001$) indicate significant differences between replicate strains.

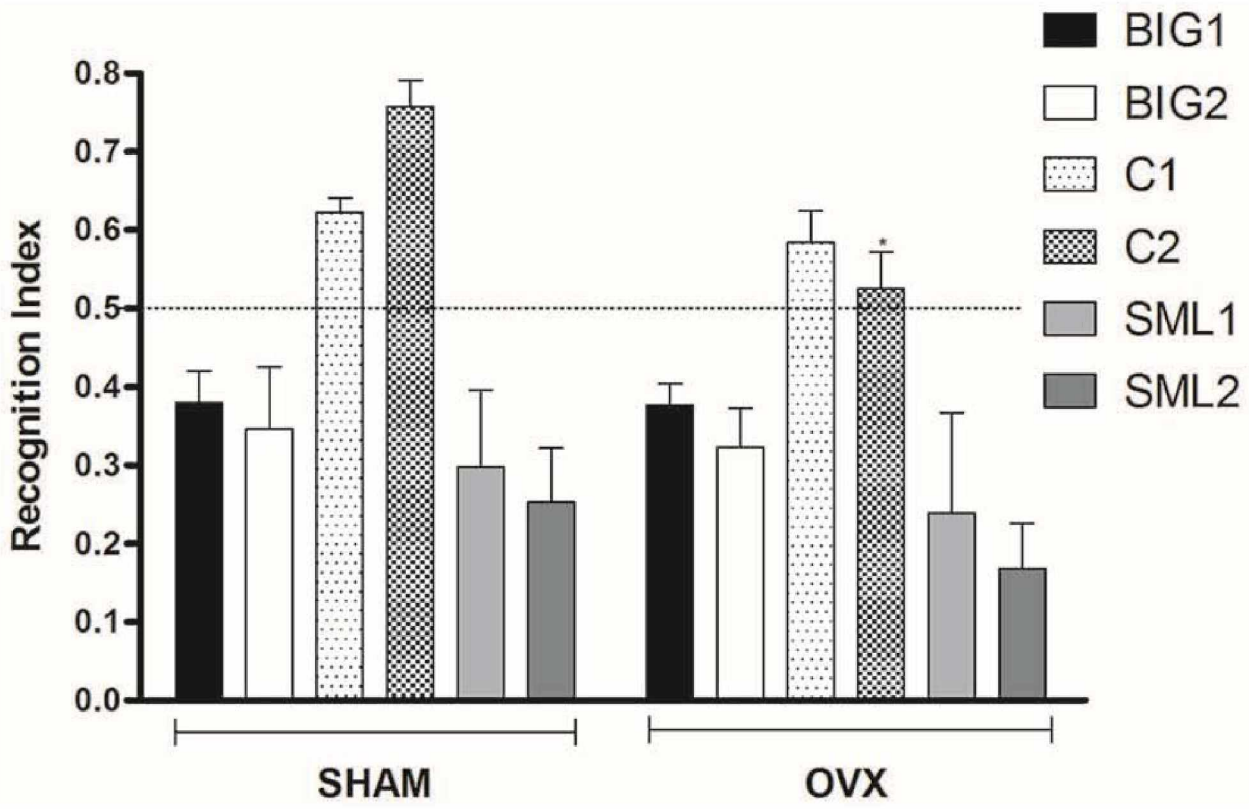

Fig 4.3 Novel object recognition in BIG, SMALL and Control strains.

The data represent the mean (± SEM) for the recognition index (RI) in the novel object recognition test between sham and OVX groups of the two replicates of the BIG, SMALL and Control strains. *($p < 0.05$) indicates significant differences between sham and OVX groups.

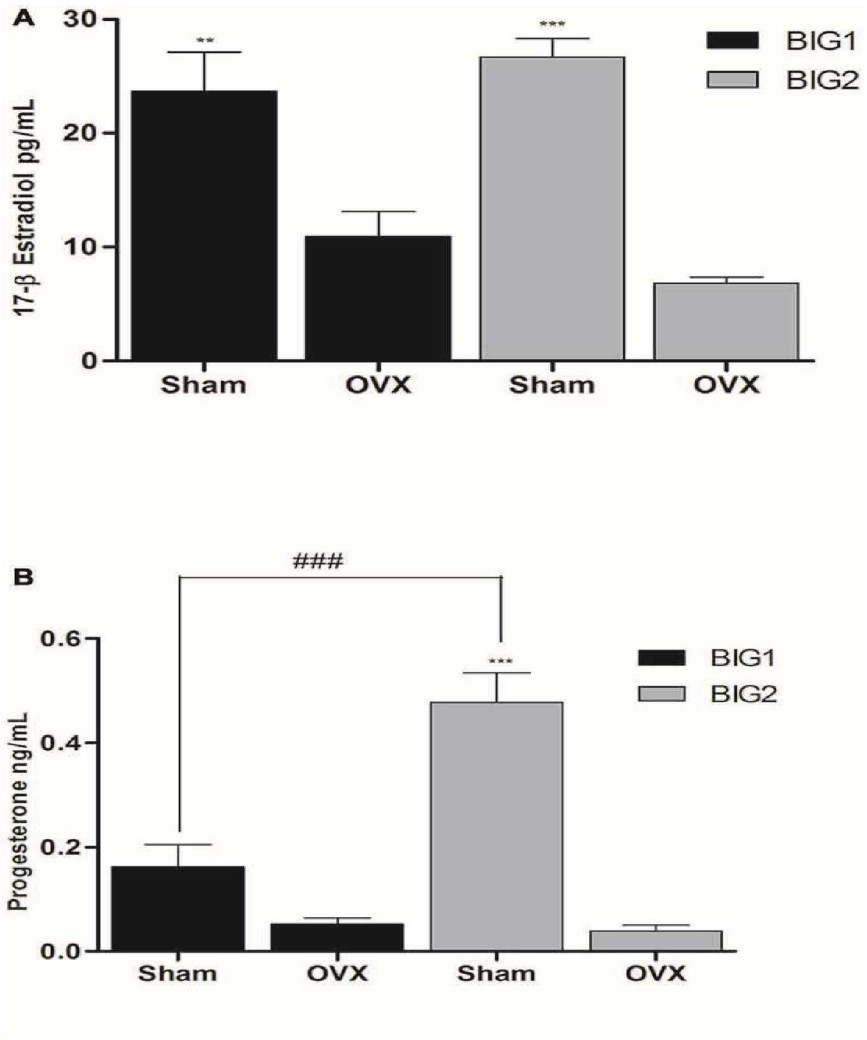

Fig 4.4 Ovarian E2 and P4 plasma levels in BIG strains.

The data represent the mean (± SEM) for plasma (A) 17β-estradiol (E2) levels and (B) progesterone (P4) levels of the BIG1 and BIG2 strains. **($p < 0.001$) and ***($p < 0.0001$) indicates significant differences between sham and OVX groups. ###($p < 0.0001$) indicate significant differences between replicate strains.

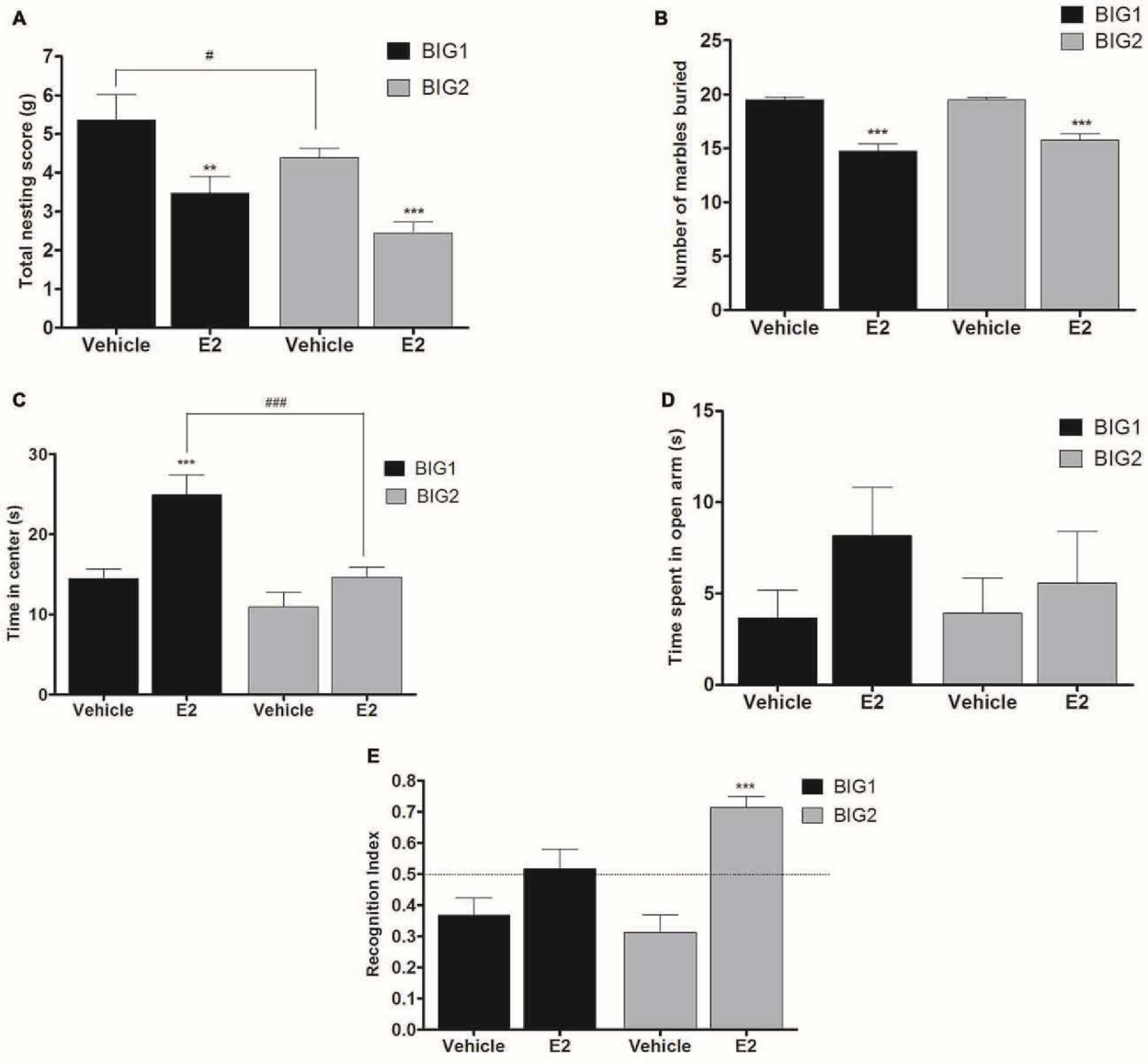

Fig 4.5 E2 administration in OVX mice.

The data represent the mean (± SEM) for (A) nesting score in grams, (B) number of marbles buried, (C) time in center in open field, (D) time spent on open arms in elevated plus maze and (E) RI in novel object recognition of the BIG1 and BIG2 strains. **($p < 0.001$) and ***($p < 0.0001$) indicates significant differences between vehicle and E2 treatment groups. #($p < 0.05$) and ###($p < 0.0001$) indicate significant differences between replicate strains.

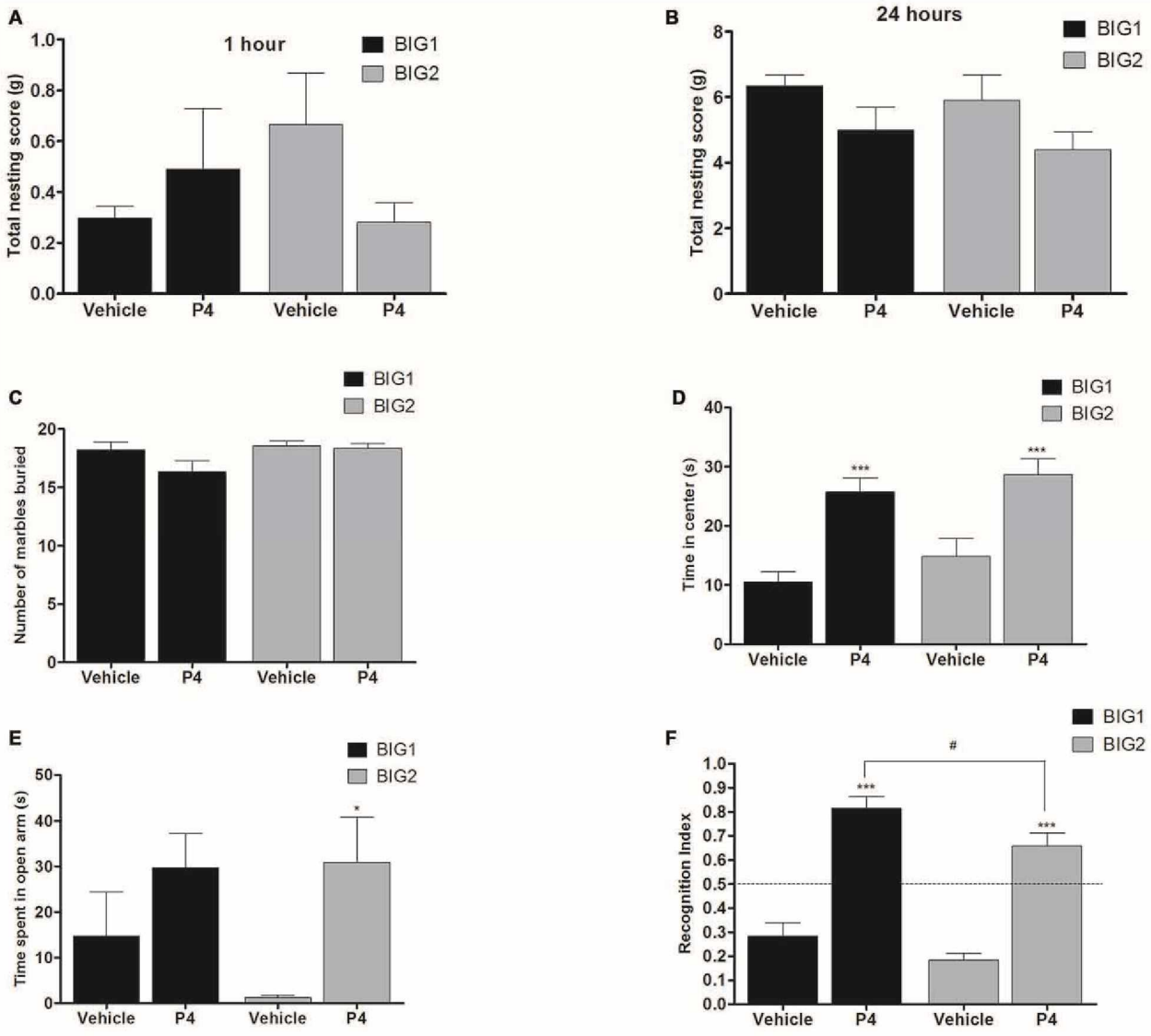

Fig 4.6 P4 administration in OVX mice.

The data represent the mean (± SEM) for (A) nesting score in grams between 0–1 h, (B) nesting score in grams between 0–24 h, (C) number of marbles buried, (D) time in center in open field, (E) time spent on open arms in elevated plus maze and (F) RI in novel object recognition of the BIG1 and BIG2 strains. *($p < 0.05$) and ***($p < 0.0001$) indicates significant differences between vehicle and P4 treatment groups. #($p < 0.05$) indicate significant differences between replicate strains.

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Chapter 5: Postpartum Lactation-Mediated Behavioral Outcomes in a Spontaneous Mouse Model of OCD is Partly Regulated by Oxytocinergic Mechanisms.[4]

5.1 Abstract

Using a spontaneous mouse model of obsessive-compulsive disorder (OCD), we investigated the compulsive-, anxiety- and depression-like behavioral responses among lactating, non-lactating and nulliparous females. Compulsive-like lactating mice were less compulsive-like in nest building and marble burying and showed enhanced responsiveness to fluoxetine (50 mg/kg) in comparison to compulsive-like non-lactating and nulliparous females. Lactating mice were however, more anxiety-like in the open field test compared to the nulliparous females, while chronic fluoxetine reduced anxiety-likes behaviors. Blocking the oxytocin receptor with L368-899 (5 mg/kg) in the lactating mice exacerbated the compulsive-like and depression-like behaviors. The dopamine D2 receptor (D2R) agonist bromocriptine (10 mg/kg) suppressed marble burying, nest building and central entries in the open field, but because it also suppressed overall locomotion in the open field, activation of the D2R receptor may have inhibited overall activity nonspecifically. Lactation- and fluoxetine-mediated behavioral outcomes in compulsive-like mice, therefore, appear to be partly regulated by oxytocinergic mechanisms. Serotonin immunoreactivity and serum levels were higher in lactating compulsive-like mice compared to non-lactating and nulliparous compulsive-like females. Together, these results suggest that the role of lactation in symptom expression and SSRI effectiveness in OCD patients requires further investigation.

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5.2 Introduction

Obsessive compulsive disorder (OCD) is one of the most prevalent neuropsychiatric disorders that affects 1-3% of the population[1]. OCD causes significant interference with quality of life which leads to functional impairments[2], disability, increased use of healthcare services and financial difficulties[3a, b]. Mood alterations and psychopathology in the postpartum condition can adversely impact both the mother and the child[4]. In females, the onset and exacerbation of obsessive-compulsive disorder (OCD) symptoms are associated with the premenstruum, pregnancy and postpartum periods[5a, b, c, d, e, f, g]. In addition, mood disorders and depression, which are often co-morbid conditions in OCD[6], precipitate during the postpartum phase in females[7a, b]. Sex differences also contribute to the phenotypic expression, heterogeneity, and drug response variations in OCD[8a, b, c] which may be partially due to physiological states influencing the expression of OCD and related symptoms in females. Hence, a spontaneous mouse model of OCD[9a, b, c, d] is employed to explore this potential explanation.

The brain undergoes dramatic changes in neuronal mechanisms during pregnancy to accommodate parturition and lactation[10a, b]. Secretion of the hormone prolactin, which stimulates milk production, from the pituitary gland lactotrophs is regulated by tuberoinfundibular neurons of the arcuate nucleus (TIDA) in the hypothalamus that secrete dopamine[11]. Dopamine secreted from the TIDA neurons acts on the D2 (dopamine type 2) receptor (D2R) on lactotrophs causing inhibition of prolactin secretion[12]. Oxytocin is produced and released in the posterior pituitary gland in response to suckling. Suckling leads to stimulation of the paraventricular nucleus (PVN) and supraoptic nucleus (SON) regions of the hypothalamus, which in turn signal more oxytocin production and release[13a, b, c]. Oxytocin facilitates contraction of the cells surrounding the alveoli in the mammary glands by binding to its receptor (OXTR) causing milk flow through the duct system[14a, b].

In addition, a novel serotonergic biosynthetic system in the mammary glands has been shown to be up-regulated during late pregnancy and lactation[15]. This is consistent with higher serum levels of serotonin in lactating female C57BL6/J mice[16]. Lactation also resulted in lower serotonin reactive serotonergic neurons in the dorsal raphe nucleus (DRN) of the brain, while behavioral responsiveness to SSRIs for anxiety-like and depression-like behaviors in lactating C57BL6/J females was enhanced when compared to virgin females[16]. Therefore, a possible interaction between the central and peripheral serotonin systems that results in modulation of behavior has been proposed[16]. Moreover, serotonergic system has also been shown to influence prolactin secretion[17a, b]. These findings suggest that hormones that regulate pregnancy and postpartum also affect serotonergic systems, which play a role in the expression of compulsive-like behaviors[9a, 18].

These unique neuroendocrinological events as a result of lactation is known to produce profound effects on healthy lactating mothers which include reduced stress, positive mood states and less anxiety[19a, b, c, d, e, f]. However, it is currently not well understood if a similar behavioral repertoire is observed in patients with predisposition to psychiatric conditions such as, OCD. Studies in rodents have indicated that postpartum phase contributes to a variety of maternal care and intruder based aggression behaviors[20a, b, c, d]. Assessments of anxiety-like behaviors, however, are contradictory as some studies have shown the postpartum phase to increase these behaviors[16, 21] while others have shown the opposite[22a, b] or no effect [16, 21-23a, b]. Studies on depression-like and compulsive-like behaviors are limited, where lactation has been shown to abolish induced compulsive-like behaviors[18] and reduce depression-like behaviors[16].

The dopaminergic and the serotonergic system on the other hand has garnered substantial support in relation to their role in OCD (reviewed in:[24a, b, c, d]) anxiety (reviewed in:[25a, b]) and depression (reviewed in:[26a, b]). Drugs that target serotonin transporters, such as selective serotonin reuptake inhibitors (SSRIs), have been effective in treating both OCD and postpartum

depression[27a, b, c, e, f, g]. Both oxytocin and prolactin which are typically upregulated in lactation has been implicated in increasing maternal care and aggression, while decreasing anxiety and depression in human[28] and animal studies[28a, b-29a, b, c, d, e, f, g, h]. However, their role in obsessions and compulsions remains inconclusive in human studies. Some studies have linked central[30] and peripheral[31] oxytocin levels to OCD, while others found no significant correlation among endogenous oxytocin levels[32] or exogenous administration of oxytocin on OCD symptomology[33a, b]. Plasma prolactin levels in response to serotonin 5HT2C receptor agonist mCPP among OCD patients has been contradictory with one study showing higher levels[30-34a, b] and another study finding the opposite[34a]. Interestingly, 8-OHDPAT-induced compulsive-like spontaneous alternation was abolished in the postpartum lactation phase of rats[18]. Hence, probing the neuropeptide/transmitter modulation during the postpartum phase in compulsive-like female mice can unravel critical neurobiological mechanisms that drive differential behavioral responses during various physiological states in a compulsive-like phenotype.

In the current study, within the context provided above, we hypothesized that in spontaneously compulsive-like female mice lactation enhances responsiveness to SSRIs and protects against excessive behavioral responses, which are mediated by dopamine and oxytocin receptor mediated pathways. We predicted that lactating females display less compulsive-like, anxiety-like and depression-like behaviors compared to non-lactating and nulliparous compulsive-like female mice. We also predicted that the compulsive-like lactating mice exhibit enhanced responsiveness to the SSRI fluoxetine in all these behaviors compared to the other experimental groups. Finally, we predicted that blocking of D2Rs decreases, while blocking of OXTRs increases compulsive-like, anxiety-like, and depression-like behaviors in lactating compulsive-like females.

5.3 Results and Discussion

5.3.1 Postpartum Lactating Compulsive-Like Female Mice Were Less Compulsive When Compared to the Non-lactating and Nulliparous Females and Exhibited Trait Specific Enhanced Responsiveness to Fluoxetine.

Lactating females buried significantly less marbles in weeks 1, 2 and 3 (Fig 5.1) when compared to the non-lactating and the nulliparous female mice ($F_{2,66}$ = 99.80, $p<0.001$). The fluoxetine treatment effect was also significant ($F_{1,66}$ = 204.70, $p<0.001$). In the presence of fluoxetine, attenuation in marble burying was observed for lactating females in weeks 1 (t_{22}= 4.651, $p<0.001$), 2 (t_{22}= 8.001, $p<0.001$) and 3 (t_{22}= 22.84, $p<0.001$). The non-lactating and nulliparous females showed no attenuation effect due to fluoxetine in week 1 (Fig 5.1a). Non-lactating females showed a decrease in marble burying in weeks 2 (t_{22}= 2.830, $p<0.001$) (Fig 5.1b) and 3 (t_{22}= 7.210, $p<0.001$) (Fig 5.1c), while nulliparous females showed a response to fluoxetine only in week 3 (t_{22}= 10.41, $p<0.001$) (Fig 5.1c). This accounted for the physiological status of the female by treatment interaction effect ($F_{2,66}$ = 34.29, $p<0.001$), indicating that lactating females had higher responsiveness to fluoxetine for marble burying when compared to non-lactating and nulliparous females.

For the compulsive-like nest building behavior (Fig 5.3), lactating females had significantly lower nesting scores in comparison to the non-lactating and the nulliparous females ($F_{2,66}$ = 125.62, $p<0.001$). The fluoxetine treatment effect was also significant ($F_{1,66}$ = 74.53, $p<0.001$). Lactating females in the fluoxetine group had lower nesting scores in weeks 1 (t_{22}= 4.473, $p<0.001$), 2 (t_{22}= 4.247, $p<0.001$) and 3 (t_{22}= 4.479, $p<0.001$) when compared to the vehicle group (Fig 5.3a, b, c). The non-lactating (t_{22}= 3.113 $p<0.01$) and the nulliparous (t_{22}= 4.280, $p<0.001$) females showed a treatment effect against the respective vehicle groups only in week 3 (Fig 5.3c). Though there was a strong trend in enhanced responsiveness to fluoxetine

by the lactating females, the physiological status of the female by treatment interaction effect was not significant ($F_{2,66} = 2.53$, $p>0.05$).

The reduced compulsive-like marble burying and nest-building in lactating compulsive-like mice aligns with a prior study in rats where 8-OHDPAT-mediated compulsive-like spontaneous alternation in T maze was abolished in the lactation phase[18]. Chronic fluoxetine treatment resulted in a greater attenuation of compulsive-like marble burying behavior in the lactating females in comparison to the non-lactating and nulliparous females. This greater responsiveness to fluoxetine observed in compulsive-like marble burying behavior also showed a trend in the compulsive-like nest-building behavior, though not statistically significant. This is an interesting finding since an unpublished study from our lab has shown that the compulsive-like mouse strain can exhibit trait specific responses to SSRI treatment. Hence, a trait specific enhanced responsiveness to fluoxetine cannot be ruled out where lactation was anti-compulsive for both marble burying and nesting (in absence of fluoxetine), but the fluoxetine response varied between the two compulsive-like behaviors. Whether enhanced responsiveness to SSRI treatment in the compulsive-like mice for one trait over the other represents clinical heterogeneity in postpartum OCD patients remains to be elucidated. The non-lactating females on the other hand did not exhibit an enhanced responsiveness to fluoxetine in compulsive-like behaviors, indicating that compulsive-like behaviors and drug efficacy in the postpartum phases can be influenced by the state of lactation and not pregnancy per se[16]. A study comparing postpartum OCD patients and normal postpartum females revealed that rates of breastfeeding was lower in OCD patients which was concomitant with precipitation of marital distress, depression and lack of social interactions[35]. Though this study[35] did not assess the effect of breastfeeding on expressions of OCD and co-morbid symptomology, it is plausible that breastfeeding can improve clinical OCD symptoms as seen with other studies on post natal anxiety where breastfeeding has been implicated as an anti-anxiety factor[36].

Various neurobiological possibilities may be contributing to this enhanced responsiveness. SSRIs are known to increase oxytocin release in humans[37], which along with the oxytocin surge due to suckling action, might provide a possible explanation for greater responsiveness to fluoxetine by lactating females[16]. Alternatively, it has been postulated that 5-HT1A autoreceptors are desensitized both by SSRIs[38a, b] and lactation specific modulation of oxytocinergic or prolactinergic systems[39a, b]. Hence, a concerted action of lactation specific events and SSRI-mediated modulation of the serotonergic system might explain the enhanced responsiveness of lactating females when compared to non-lactating and nulliparous females.

The reduced compulsive-like behavior is contrary to a previous study in which lactating wild type C57BL6/J females treated with daily vehicle injections exhibited more marble burying behavior than nulliparous females[16]. Considering the low number of marbles buried, i.e., about 3 in nulliparous and about 7 in lactating females on average in lactating females during the 20-min test[16], C57BL6/J mice can be considered non-compulsive-like mice[9a]. May be the lower rates of marble burying reflect anxiety-like behavior while higher rates of digging better reflect a compulsive-like phenotype as seen in our mouse strains[9a, b, c, d]. Alternatively, the contradictory effect of lactation on compulsive-like digging may indicate a strain effect due to genetic differences as a result of selection for expression of compulsive-like phenotype or different genetic correlation structures due to founder effects or random genetic drift[40]. Hence, the results provide insight into how genetic variability among healthy and OCD patients could produce differential outcomes in compulsive-like behavior during postpartum.

5.3.2 Administration of OXTR Antagonist Exacerbated Compulsive-Like Behaviors in Lactating Compulsive-Like Female Mice.

Administration of OXTR antagonist, L368-899 increased compulsive-like marble burying ($F_{2,28}$ = 76.74, $p<0.001$) in the lactating females when compared to the vehicle (t_{18}= 5.305, $p<0.001$) and D2 agonist, bromocriptine (t_{19}= 12.33, $p<0.001$) treated lactating females (Fig 5.2).

For compulsive-like nest-building behavior there was an overall treatment effect for hours 1 ($F_{2,31}$ = 117.42, p<0.001), 2 ($F_{2,31}$ = 52.54, p<0.001), 3 ($F_{2,31}$ = 14.05, p<0.001), 5 ($F_{2,31}$ = 9.36, p<0.001) and 24 ($F_{2,31}$ = 66.56, p<0.001) (Fig 5.4a, b). The drug effect in hours 1 and 2 was due to lactating females treated with L368-899 showing higher nesting scores than the lactating females treated with vehicle (t_{21}= 6.260, p<0.001; t_{21}= 5.102, p<0.001) or bromocriptine (t_{21}= 7.307, p<0.001; t_{21}= 7.603, p<0.001). Significantly higher nesting scores for vehicle (t_{20}= 5.528, p<0.001) and L368-899 (t_{20}= 4.509, p<0.001) treated lactating females when compared to bromocriptine treated females accounted for the drug effect in hour 3. Drug effect in hour 5 was due to an increase in the nesting scores of bromocriptine treated animals in comparison to vehicle (t_{20}= 4.173, p<0.001) and L368-899 (t_{21}= 4.081, p<0.001) treated females. After 24 hours the overall nesting scores were significantly higher in L368-899 treated lactating females in comparison to the vehicle (t_{21}= 5.579, p<0.001) or bromocriptine (t_{21}= 4.473, p<0.001) treated ones (Fig 5.4b).

The current data indicates that blocking OXTR during lactation in compulsive-like mice can significantly exacerbate compulsive-like behaviors. The neuropeptide oxytocin has been investigated in connection to OCD in clinical studies[30-33]. However, the association of oxytocin and OCD has been contradictory[30-33a, b]. OXTR on the other hand, is widely distributed in various brain regions[14b,41a, b], which is suggestive of its wide range of effects in the CNS[29f]. A study comparing human OCD patients with controls linked OXTR epigenetic modulation to OCD severity[42].

The current finding of the role of oxytocinergic modulation during postpartum lactation in animal models that exhibit compulsive-like phenotype is novel. Emerging evidence from rodent studies suggest that oxytocinergic and serotonergic systems interact with each other to regulate behavioral responses in a context dependent manner[29f, 43a, b, c]. For example, oxytocin produced anxiolytic effects in mice by regulating serotonin transmission through OXTR expressed on

raphe neurons[29f]. This serotonin release and anxiolytic response was positively regulated by 5-HT2A/2C receptors[29f]. OXTR deletion on raphe neurons influenced aggression in a sex dependent fashion with male mice exhibiting enhanced aggression and females exhibiting no changes in aggression[43a]. This oxytocinergic and serotonergic interaction has also been reported during lactation[39] in addition to the lactation specific extensive neuro-glia remodeling of the oxytocinergic systems[10a, 16]. Moreover, spontaneous alternation due to activation of 5-HT1A receptor by 8OH-DPAT was abolished during lactation in rats[18]. One of the likely explanations that the authors proposed for this response was desensitization of the 5-HT1A receptor due to oxytocinergic or prolactinergic modulation specific to lactation[18, 39, 44a, b]. The current result from our study is suggestive of a possible interaction of oxytocinergic and serotonergic systems to exhibit behavioral modulation in compulsive-like mice during postpartum lactation. However, to what extent this interaction is involved in regulating spontaneous compulsive-like behavior remains to be determined.

Attenuation of marble burying and nesting behavior observed in bromocriptine treated lactating females was most likely due to the overall behavioral suppression as locomotion was suppressed in the open field (see below). This was unexpected, since we selected a dose which was used in a prior study with mice and did not influence motor behaviors[45], which suggests that compulsive-like may be more sensitive to bromocriptine. Further, in another study, a bromocriptine dose of 32 mg/kg evoked climbing behavior in mice[46]. It should be considered that the acute effect of dopamine agonists on locomotory functions is biphasic with initial depression followed by excitation[47a, b], which the lactating compulsive-like mice also displayed for nest-building behavior. The behavioral depression due to bromocriptine in the compulsive-like lactating females could be attributed to the stimulation of the presynaptic dopamine D2Rs[48a, b] in brain regions implicated in motor functions such as dorsal striatum and nucleus accumbens[49].

5.3.3 Postpartum Lactating and Non-Lactating Compulsive-like Female Mice Exhibited Anxiety-Like Behavior in the Open Field when Compared to Nulliparous Females.

For the anxiety-like behavior in the open field, significant physiological status and fluoxetine treatment effects on the central zone entries were observed ($F_{2,64}$ = 8.52, $p<0.01$ and $F_{2,64}$ = 22.03, $p<0.001$, respectively) (Fig 5.5a). Compulsive-like lactating females had significantly lower central zone entries when compared to nulliparous females in the vehicle group (t_{22}= 2.591, $p<0.05$, respectively). In the fluoxetine treated groups, the lactating females showed less number of central entries (t_{21}= 3.213, $p<0.001$) when compared to the nulliparous females. The significant treatment effect was due to the improvement in the central square entries for fluoxetine treated lactating (t_{21}= 2.532, $p>0.05$) and nulliparous (t_{22}= 3.307, $p>0.05$) females when compared to their vehicle treated counterparts. This was not seen for the non-lactating females. There was no significant effect of physiological status ($F_{2,64}$ = 1.72, p=0.18), treatment ($F_{1,64}$ = 2.59, p=0.11) and physiological status by treatment interaction $F_{2,64}$ = 1.12, p=0.33) effects on locomotor activity, measured as total distance traveled (Fig 5.5b).

Contrary to our hypothesis, the data indicates that lactating compulsive-like mice were more anxiety-like in comparison to nulliparous ones. Lower levels of anxiety during postpartum is considered to be a maternal adaptation due to neuroendocrine modulation[50a, b], which facilitates social bonding and offspring acceptance[51a, b]. However, the component of emotionality can be perceived very differently by lactating females as evaluated through behavioral tests. For example, evaluation of anxiety behavior between postpartum and virgin mice and rats in the anxiety-like light-dark test revealed no differences[23a, b], while locomotion was reduced in lactating rats when compared to virgin ones[52]. Further, when isolated from pups lactating wild type C57BL/6 mice were found to be more anxious in marble burying behavior[16]. It is interesting to note that though lactating females were more anxiety-like, locomotory activity among vehicle treated lactating, non-lactating and nulliparous compulsive-like females were not different, which

supports that the anxiety-like differences were not due to activity level differences. Hence, motivation to explore novel environment was not affected in the lactation phase. Alternatively, selective breeding for compulsive-like phenotype could have resulted in greater anxiety in our mouse strains during the postpartum lactating phase. It could be due to a genetic correlation between genes that affect compulsivity and postpartum anxiety or different genetic correlation structures due to founder effects or random genetic drift[40]. Further, strain specific anxiety-like behavioral differences have been observed in rodents[53a, b].

Though improvement in the number of central entries was observed in lactating and nulliparous females following fluoxetine treatment, no significant effect was seen for the non-lactating females (although it trended in the same direction). It points towards a lesser response to fluoxetine in the compulsive-like non-lactating mice. This is interesting since, the state of lactation or the presence of pups has been proposed to be an important factor in influencing SSRI responsiveness in a study with C57BL6/J[16]. The fact that both lactating and nulliparous compulsive-like mice responded to fluoxetine, indicates that the state of lactation is not the only factor that drives SSRI responsiveness for anxiety-like behavior. However, we believe that removal of pups and therefore cessation of lactation can result in reduced responsiveness to SSRIs for anxiety-like behaviors. The current data could provide enhanced understanding as to how OCD patients might vary in their emotional responses during postpartum with potentially greater vulnerability for lactating females and poor response to SSRI for non-lactating ones.

5.3.4 Blocking OXTR Did Not Affect Anxiety-Like Open Field Behaviors, While Activating D2 Receptors Through Bromocriptine Increased Anxiety-Like Behavior and Suppressed Overall Activity in Lactating Compulsive-Like Females.

A significant treatment effect was observed for the number of central entries ($F_{2,29} = 7.60$, $p<0.01$) (Fig 5.6a) and total locomotion ($F_{2,29} = 12.43$, $p<0.001$) (Fig 5.6b) in the anxiety-like open field measure. This was mainly due to the suppression of total locomotion in the

bromocriptine treated lactating females that also contributed to a lower number of central entries when compared to the vehicle (t_{19}= 3.243 $p<0.01$) and L368-899 (t_{20}= 4.924 $p<0.001$) treated lactating females. No significant differences were observed for number of central entries (t_{20}= 0.0393 $p>0.05$) and locomotion (t_{20}= 1.722 $p>0.05$) between vehicle and L368-899 treated females.

OXTR did not alter the anxiogenic response of lactating compulsive-like mice. Lack of effect on anxiety-like behavior due to blocking of OXTR is in agreement with other rodent studies where OXTR antagonists did not cause differences in various anxiety-like assessments[54a, b]. Further, a study with OXTR knockout mice did not reveal significant differences in the anxiety-like measures from wild type controls[51b]. However, it has to be considered that these studies were not conducted in the postpartum phases. In fact, a study in rats showed that blocking OXTR can produce anxiogenic behaviors in pregnant and lactating females when compared to virgin females[55]. A likely explanation for this differential response could be due to low oxytocin system activity during non-reproductive and stress-free events[56]. This indicates that the oxytocinergic system might not maintain basal levels of anxiety-like responses but can regulate such behaviors during specific physiological conditions such as pregnancy and lactation[56]. Studies on wild type mice have shown that chronic intracerebroventricular infusions or nasal application of oxytocin resulted in downregulation of oxytocin receptors in brain regions regulating anxiety[57a] probably due to OXTR desensitization[58]. Interestingly, chronic oxytocin treatment upregulates vasopressin receptor binding in the lateral septum where vasopressin is known to exert anxiogenic effects[57b, 59]. An oxytocin surge during lactation that desensitizes OXTR in specific brain regions could therefore have increased anxiogenic behavior in the lactating compulsive-like mice. In that scenario, blocking OXTR would not be expected to alter anxiety-like behavior as observed in the compulsive-like lactating females indicating no role of OXTR in driving anxiety-like responses during lactation. Though

oxytocin is known to reduce anxiety-like responses for maternal care[60], it could be one of the many factors contributing towards these responses[61]. The anxiogenic behavior observed in bromocriptine treated lactating females was predominantly due to the overall suppression of locomotory activity as discussed in the context of compulsive-like behavior.

5.3.5 Physiological Status in Fluoxetine Treated Compulsive-Like Female Mice Determined Depression-Like Tail Suspension Behavior.

For depression-like tail suspension behavior a significant effect of the physiological status ($F_{2,64}$ = 8.38, $p<0.001$) was found in the compulsive-like mice (Fig 5.7a). There were no significant treatment ($F_{1,64}$ = 0.14, p=0.71) and physiological status by treatment interaction ($F_{2,64}$ = 1.60, p=0.21) effects. The physiological effect was primarily due to lactating (t_{20}= 3.840, $p<0.001$) and non-lactating (t_{22}= 2.692, $p<0.001$) females having lower immobility in fluoxetine treated groups when compared to fluoxetine treated nulliparous females.

Overall, the data indicates that physiological status of the compulsive-like mice did not influence depression-like behavior under normal conditions. However, in the presence of fluoxetine, depression-like behavior increased in the nulliparous mice when compared to the fluoxetine treated non-lactating and lactating compulsive-like females. The data also shows that fluoxetine did not attenuate depression-like behavior in the non-lactating, lactating and nulliparous compulsive-like mice. In an inbred mouse strain lactating females exhibited less depression-like behavior[16]. In another study, chronic treatment of fluoxetine (SSRI) in wild type mice exposed to chronic stress conditions exacerbated the depression-like behavior when compared to mice exposed to enriching conditions which improved performance in depression-like behavior[62]. We believe that the responses of the lactating, non-lactating and nulliparous compulsive-like mice in the fluoxetine group is probably due to the selective breeding that resulted in a higher stress response[9c]. This might be compounded by physiological events, such as postpartum, accounting for lack of antidepressant effect in the presence of fluoxetine. OCD

patients, especially females and postpartum females, exhibit an enhanced HPA axis and a higher baseline stress response[63a, b], which could lead to reduced SSRI effectiveness in treating depression among postpartum patients.

5.3.6 OXTR Antagonist, L368-899 Increased Depression-Like Behavior in Lactating Compulsive-Like Females, While the D2 receptor Agonist Bromocriptine Had No Effect.

An overall treatment effect ($F_{2,64}$ = 8.38, $p<0.001$) was observed for lactating females undergoing tail suspension behavior (Fig 5.7b). Lactating females administered with L368-899 had higher immobility in the tail suspension when compared to vehicle (t_{16}= 5.110, $p<0.001$) and bromocriptine (t_{16}= 4.500, $p<0.001$) administered females.

This data indicates that blocking OXTR increased depression-like behavior in compulsive-like lactating females. The antidepressant activity of oxytocin has been established in rodent models[64a, b, c] and is facilitated through OXTR[65]. Oxytocin is known to control stress adaptation by projecting from the paraventricular region of the hypothalamus to areas like the amygdala, hippocampus and lateral septum (reviewed in:[66]). This is also in good agreement with the OXTR expression profile in these regions (reviewed in:[14b]). Hence, blocking OXTR in our study decreased immobility time in the tail suspension test.

Interestingly, bromocriptine did not depress mobility in the tail suspension test compared to vehicle, although it was measured at the same time after injection as the open field test. Bromocriptine reduces immobility of mice in the tail suspension test[67a, b] and has been shown to have antidepressant effects in preclinical and clinical studies[68a, b, c, d]. Therefore, this anti-depressant effect of bromocriptine may have compensated for the lower levels of activity observed for the bromocriptine treated lactating compulsive-like mice in the marble burying, nest building, and open field tests. Alternatively, activation of the D2R by bromocriptine reduced

compulsive-like behaviors, made the lactating compulsive-like female mice more anxiety-like, reduced locomotor activity, but had no effect on depression-like behavior.

5.3.7 Lactating Compulsive-like Females Exhibited Higher DRN Serotonin (5-HT) Immunoreactvity and Serum Levels When Compared To Non-lactating and Nulliparous Females.

The physiological status effect was significant on the total number of 5-HT positive neurons in dorsal raphe nucleus ($F_{2,12}$ = 15.30, $p<0.001$). Lactating females had higher 5-HT immunoreactivity when compared to the non-lactating (t_8= 6.788, $p<0.01$) and nulliparous (t_8= 6.763, $p<0.001$) females (Fig 5.8). The serum levels of 5-HT in lactating females were also significantly higher ($F_{2,15}$ = 5.56, $p<0.05$) in comparison to the non-lactating (t_{10}= 4.161, $p<0.05$) and nulliparous females (t_{10}= 4.000, $p<0.05$) (Fig 5.9).

The higher level of serum 5-HT levels in lactating compared to non-lactating compulsive-like female mice is in agreement with a similar finding in C57BL/6 mice[16]. Peripheral 5-HT is predominantly produced in the enterochromaffin cells of the gut that enter into the blood stream from where they get transported into platelets[69a, b]. High peripheral 5-HT levels are typically seen during pathologies such as tumors[16]. lactation is considered to be a unique physiological state[16]. Two probable sources of 5-HT during lactation could account for the observed high serum 5-HT levels in lactating females. One source of higher 5-HT could be due to enhanced 5-HT synthesis in the mammary glands during lactation[15], while another possibility could be due to elevated mucosal hyperplasia in the intestine of lactating females[16, 70].

The increased DRN 5-HT immunoreactivity in lactating females versus decreased 5-HT immunoreactivity in non-lactating and nulliparous females is indicative of altered central serotonergic activity during lactation. However, this finding was opposite of what was found in c57BL/6 mice, in which lactating females had decreased DRN 5-HT immunoreactivity compared

to virgin females[16]. The discrepancy in DRN immunoreactivity could be due to the collection of brain tissues at different time points during the lactation period, i.e., 10 days after parturition[16] and 18 days after parturition in our study. Alternatively, selective breeding for compulsive-like phenotype might have resulted in higher 5-HT immunoreactivity when compared to nulliparous or non-lactating mice. The reduction in DRN 5-HT immunoreactivity in the c57BL/6 lactating mice[16] could also reflect increased activity of the serotonergic system due to greater release accounting for the anxiolytic and antidepressant behaviors. On this premise, the increased DRN 5-HT immunoreactivity in the compulsive-like lactating females might reflect decreased activity of the serotonergic system which aligns with the anxiogenic behaviors exhibited by the lactating females. We have shown previously that the compulsive-like mice had lower numbers of arginine-vasopressin (AVP) immunoreactive neurons in the suprachiasmatic nucleus (SCN) compared to non-compulsive-like mice[71]. This difference in immunoreactivity was abolished when AVP transport and release was blocked by colchicine[72]. In organotypic SCN cultures, we also showed that compulsive-like mice had higher levels of AVP release per AVP neuron compared to the non-compulsive-like mice[72]. Therefore, a lower immunoreactivity of AVP in the SCN indicated a more active AVP system, which is similar to what might have been the case with serotonergic system in relation to DRN 5-HT immunoreactivity. This concept of higher immunoreactivity correlating with lower release and vice versa has been corroborated in other studies with neuropeptides[73a, b]. Finally, the possibility of higher serotonin immunoreactivity could be related to higher serotonin activity at synapses, thereby explaining lower compulsive-like nest-building and marble burying behaviors in the lactating females when compared to non-lactating and nulliparous females. Depleting serotonin in the brain by knocking out tryptophan hydroxylase 2, the initial rate-limiting enzyme in serotonin synthesis, increased compulsive-like behaviors, including nestlet shredding and marble burying[74], compared to wild-type mice, which indicates that activation of the serotonergic system is necessary to reduce compulsive-like behaviors.

5.4 Conclusion

The current study provides a novel understanding of the role of physiological status on treatment and behavioral outcomes in the female compulsive-like mouse model, in which lactation protected compulsive-like female mice from expressing compulsive-like behaviors and increased their response to the SSRI fluoxetine in reducing compulsive-like behaviors. This study also revealed that the oxytocinergic system during postpartum lactation was at least partially responsible for mediating these effects of lactation on mice with a compulsive-like phenotype. Considering a pressing need for better understanding of OCD in females during physiologically challenging conditions, our data holds clinical importance by providing critical insights into SSRI effectiveness, role of breast feeding and comorbid affective disorders in postpartum OCD patients.

5.5 Materials and Methods

The University of Alaska Fairbanks Institutional Animal Care and Use Committee approved the animal care and experimental procedures (IACUC assurance number 862663).

5.5.1 Animals

For this study we used the compulsive-like BIG male and female mice[9]. This compulsive-like mouse model was developed from house mouse strains (Mus musculus) through bidirectional selection for nest-building behavior[40, 75]. The HS/Ibg outbred strain[76], which was developed through crossing of eight inbred strains (A, AKR, BLB/c, C3H/2, C57BL, DBA/2, Is/Bi, and RIII) served as the stock population for the selective breeding[75]. Bidirectional selection resulted in two compulsive-like BIG strains, which consistently exhibit a forty-fold higher level of compulsive-like nest-building behavior[40, 75] and a three-fold higher level of compulsive-like marble burying behavior [9a] when compared to the two non-compulsive-like SMALL strains. The two randomly-bred control strains express intermediate levels of these behaviors[9a, 9c, 40, 75]. This excessive, repetitive and perseverant otherwise normal, nest-building and marble burying

behavior by the BIG strains makes them a good model to study compulsive-like phenotypes[9]. All experimental BIG males and females, taken from the one compulsive-like strain that had the highest breeding success of about 90% during colony breeding, were housed in polypropylene cages (27 cm × 17 cm × 12 cm) with same sex littermates and provided with wood shavings under a 12:12 light-dark cycle at 22 ± 1°C with *ad libitum* food (Purina Mills, Lab Diet Mouse Diet #5015, St. Louis, MO) and water. When the animals reached 60 days of age, each BIG female was paired with a BIG male in a cage with *ad libitum* access to high protein rodent chow (Masuri Rodent Diet #5663, Purina Mills, LLC, St. Louis, MO, USA) and water.

5.5.2 Experimental Design

The entire study was divided into two main phases. In the first phase of the experiments, compulsive-like lactating, non-lactating and nulliparous female mice were tested for compulsive-like, anxiety-like and depression-like behaviors. For lactating and non-lactating females, the males were separated two weeks after pairing. All the females showed signs of pregnancy within two weeks of pairing which was confirmed through vaginal plug. All lactating females were housed with their pups until the end of all the behavioral assessments. For the non-lactating females, pups were removed immediately after birth and the females were housed individually in cages until the end of all experimentation. Nulliparous females were housed individually in cages after they failed to conceive following pairing with male counterparts for two weeks (males were removed after two weeks of pairing). All experimental groups of females (lactating, non-lactating and nulliparous) were either treated with fluoxetine (n=12) or vehicle (n=12). Animals treated with either fluoxetine or vehicle were subjected to compulsive-like marble burying and nest-building behavior once every week for three weeks on days 1, 2, 8, 9, 15 and 16 of lactation, respectively (day 0 was the day of parturition). Anxiety-like open field and depression-like tail suspension tests were conducted only once on days 17 and 18 of lactation, respectively.

Separate groups (n=5 per group) of lactating, non-lactating and nulliparous females that did not undergo any behavioral assessments and treatments were subjected to transcardial whole body perfusion on day 18 of lactation. Brains were collected for immunohistochemical analysis of 5-HT neurons in the dorsal raphe nucleus (DRN). Other separate groups of lactating, non-lactating and nulliparous females (n=6 per group) that also were not subjected to any behavioral assessments and treatments were used to collect blood samples on day 18 of lactation from the trunk of cervically dislocated animals for serum analysis of 5-HT through ELISA. Because most of the behavioral assessments (except compulsive-like behaviors, which were performed once every week) were performed in the third week, we wanted to evaluate the 5-HT serum levels and DRN immunoreactivity during the final week of lactation. Day 18 of lactation was selected for 5-HT measurements since it is considered to be the peak lactation period[77a, b].

For phase two of the experiments, only lactating females were used. All lactating females were divided into 12 groups. Animals were tested for compulsive-like nest-building and marble burying, anxiety-like open field and depression-like tail suspension. For each of four behaviors, three main treatment groups were used for a total of 12 experimental groups. For each behavior mice were treated with bromocriptine mesylate a dopamine D2 receptor agonist [78], L368-899 an OXTR antagonist[79] or vehicle. All drug or vehicle treated lactating females were subjected to a single behavioral assessment, i.e., tested just once for one behavior, on day 18 of lactation. Exposing animals to multiple behaviors were avoided to minimize stress to the pups and the females due to multiple injections. All behavioral assessments were carried out by an experimenter blinded to the treatment groups.

5.5.3 Drug Treatment

For phase one of the experiments, all groups (lactating, non-lactating and nulliparous females) were treated with either fluoxetine or vehicle. The vehicle animals received sucrose in

drinking water (2.9 g/L) while the drug groups received 50 mg/kg of fluoxetine in the sucrose vehicle as described previously[9a]. The dosage of 50 mg/kg was used because it shows the most consistent suppression of compulsive-like behaviors in our compulsive-like mice[9a]. The dosage of 50 mg/kg was used since it shows the most consistent suppression of compulsive-like behaviors in our mice[9a]. The route of administration was orally in the drinking water. The average water consumption was measured for 3-5 days before and after the pups were born in lactating and non-lactating females. Treatment for lactating and nonlactating females started on the day the pups were born. The amount of drug given was calculated based on the average body weight and daily average water consumption of the mice. The water levels were measured daily and new water bottles were given every two days. To avoid excess consumption of treatment only minimum volumes, determined through measurement of average daily water consumption, were provided and animals were checked every 12 hours. For the nulliparous females, the same time frame was used for evaluation of drug and vehicle treatments, and treatment was started when it was confirmed that they were not pregnant, i.e., three weeks after the males were removed. The total duration of treatment was 18 days because after three weeks of SSRI treatment maximum fluoxetine effects are obtained in our mouse model of OCD[9a].

In phase two of the experiments, lactating females were divided into three drug treatment groups for a total of four behaviors to a total of 12 experimental groups. Each behavioral group received 10 mg/kg of D2R agonist bromocriptine mesylate, 5 mg/kg of OXTR antagonist L368-899 or vehicle (0.9% sterile saline and 1% Tween) through intraperitoneal (i.p) injection one hour before behavioral assessment. The dosage for L368-899 and bromocriptine was determined from previous studies in mice and rats[78-80a, b, c]. The injection volume was adjusted proportionally according to the body weight of each animal with a 0.3 ml injection volume for a 40g mouse.

5.5.4 Compulsive-like Behaviors

5.5.4.1 Marble Burying Behavior

The marble-burying test was used to assess compulsive-like behavior[9a, 74, 81a, b]. All mice were individually introduced into a polypropylene cage (37 cm × 21 cm × 14 cm) containing 20 glass marbles (10 mm in diameter) evenly spaced on 5 cm deep wood shavings firmly pressed into a uniform bedding without access to food or water for 10 min. The total number of marbles buried at least 2/3 in the 10-min period was quantified as compulsive-like digging behavior[9]. Following the 10-min test, animals were returned to their home cages.

5.5.4.2 Nest-building Behavior

Nest-building behavior was used to assess the compulsive-like phenotype of the female mice[9a, b, c, d]. All mice were housed individually and were allowed to access a pre-weighed cotton roll placed in the cage top food hopper. The amount of cotton used by the mice after 1 hour was determined by weighing the cotton roll. For phase one of the experiments nest-building was performed for 1 hour only. This was mainly to ensure that the lactating females were not separated from the pups for too long. The same time frame was followed for non-lactating and nulliparous females to have uniform data collection. Nest-building was performed on weeks 1, 2 and 3 of postpartum for lactating and non-lactating females. A similar time frame was also maintained for nulliparous females and was tested thrice on weeks 1, 2 and 3.

For phase two of the experiments all lactating females treated for vehicle, bromocriptine or L368-899 were subjected to nest-building for 24 hours only once starting on day 18 of lactation. Amounts of cotton used for nesting was measured after 1, 2, 3, 4, 5 and 24 hours after i.p injection.

5.5.5 Anxiety-like Open Field Behavior

Anxiety-like behaviors were determined through the open field test[82a, b]. Mice were individually introduced into an open field (40 cm × 40 cm × 35 cm) with a central zone (20 cm × 20 cm). The apparatus was placed underneath an overhead light illuminating the entire apparatus[9a]. The animals were placed in the central zone of the apparatus and their behavior was videotaped for 3 min and analyzed with the aid of ANYMazeTM video tracking software (Stoelting Co., Wood Dale, IL, USA). The total number of central zone entries (anxiety-like measure) and total distance traveled (locomotion) in the entire open field were measured. The open field was cleaned before each test with a dilute chlorhexidine solution. Prior experiments[9] with the BIG mice indicate that 3 min duration provides consistent outcomes for assessment of locomotor activity and anxiety-like behaviors and therefore considered for the current experiment.

5.5.6 Depression-like Tail Suspension Behavior

Depression-like behavior in the compulsive-like mice was assessed through the tail suspension test[83]. Each mouse was individually suspended from a hook of the tail suspension apparatus (Stoelting Co., Wood Dale, IL) above the surface of the table by a 15 cm long adhesive tape. The tape was placed 5mm from the tip of the tail and the animal was suspended 60 cm above the base of the apparatus. The immobility duration was recorded sideways for 6 minutes using a camera. Mice were considered immobile only when they were completely motionless without any movement of body parts or front and hind limbs[84]. The videos were analyzed by an experimenter blinded to the outcome of the study.

5.5.7 Serum 5-HT Levels

Trunk blood was collected from females (lactating, non-lactating and nulliparous) on the 18th day of lactation after cervical dislocation. Blood samples were kept undisturbed at 4 degrees overnight. This allowed the 5-HT release from platelets[16]. Serum was then extracted

from blood samples by centrifugation at 12,000 rpm for 20 minutes (4 degrees). The extracted serum was stored at -80 ºC until analysis. All serum samples were assayed in duplicates for 5-HT levels using an ELISA kit (Kit# ADI: 900-175; Enzo life Sciences) according to the manufacturer instructions. The sensitivity for the kit was 0.293 ng/mL

5.5.8 5-HT Immunohistochemistry in DRN

Brains collected through whole body transcardial perfusion (8 mL of 0.01M phosphate buffer saline followed by 25mL of 4% paraformaldehyde in PBS) were fixed overnight in 4% paraformaldehyde. Fixed brains were transferred to 30% sucrose solution and stored until they submerged completely. Brains were then frozen in Tissue-Tek OCT compound in beaker cups (VWR International). Frozen brains were sectioned on a freezing microtome at 20µm coronal slices -4.84Bregma and -5.02Bregma as per the mouse brain atlas. Brain slices were then immunostained for 5-HT on slides (VWR Cat no: 48311-703). Slides containing brain sections were first washed in 1X PBS (five 5 min washes). The slides were then incubated in 30% hydrogen peroxide in PBS for 30 minutes at room temperature followed by PBS washes (six 5 min washes). Blocking buffer (1x PBS containing 5% normal goat serum, 2% BSA and 0.4% Triton X-100) was then added to the slides for one hour. After one hour, slides were washed in 1X PBS (three 5 min washes) and incubated with 5-HT primary (Immunostar Cat: 20080; 1:20,000 in 1X PBS and 0.4% Triton X-100) overnight for 20 hours. On day two, slides were washed in PBS (four 5-min washes) and were incubated with secondary antibody (1:600 biotinylated goat anti-rabbit, Vector labs Cat: BA1000; in PBS and 0.04% Triton X-100) for one hour. Sections were finally processed using Vectastain Elite ABC immunoperoxidase system (Vector Laboratories) as per the manufacturer instructions and visualized with Ni^2+-DAB enzyme substrate. For analysis of 5-HT reactive cell bodies in the DRN, ImageJ software (NIH) was used. In the threshold mode which was same for all brain section analyzed, the number of cell bodies stained positively for 5-HT in the DRN were counted.

5.5.9 Statistical Analysis

Statistical system software (SAS; Version 9.4 NC Carey) was used for analysis of all the results. For the first phase of experiments, a generalized linear model (GLM) repeated measures analysis of variance (ANOVA) for physiological status (lactating, non-lactating and nulliparous), treatment (vehicle, fluoxetine) and physiological status by treatment interaction effects was used to statistically evaluate the nest building and marble burying behaviors. A GLM two-way ANOVA for physiological status, treatment and physiological status by treatment interaction effects was used to statistically evaluate the open field and tail suspension behaviors. For serum and DRN 5-HT levels, a GLM one-way ANOVA was conducted for the effect of physiological status. For the second phase of the experiments, a GLM one-way ANOVA was performed to test for the effect of treatment (bromocriptine, vehicle and L368-899) on each of four behaviors. When significance was found in any of the ANOVA measures, appropriate pairwise comparisons were performed using the Studentized Range test. The nesting scores were square root transformed to obtain a more normal distribution[9, 40, 85a, b]. All data are represented as mean ± standard error of the mean (SEM). A probability level of $p<0.05$ was used to determine statistical significance in all cases.

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5.7 Figures

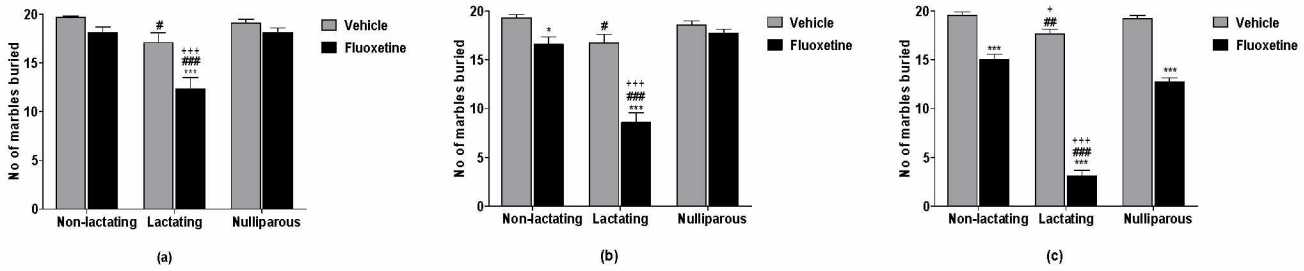

Fig 5.1 Lactating compulsive-like female mice exhibited less compulsive-like marble burying. Compulsive-like marble buying behavior represented as the total number of marbles 2/3rd buried among lactating, non-lactating and nulliparous compulsive-like females administered with vehicle or fluoxetine in (a) week 1 (b) week 2 and (c) week 3. *** ($p<0.001$) indicates significant differences between fluoxetine and vehicle groups. # ($p<0.05$), ## ($p<0.01$) and ### ($p<0.001$) indicate significant differences between lactating and non-lactating females within each treatment group. + ($p<0.05$) and +++ ($p<0.001$) indicate significant differences between lactating and nulliparous females within each treatment group. Data is expressed as the mean ± SEM.

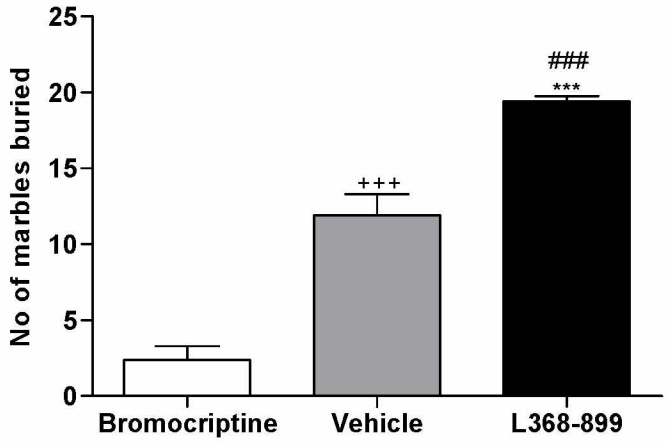

Fig 5.2 The OXTR antagonist increased compulsive-like marble burying, while the D2R agonist decreased marble burying in lactating compulsive-like female mice.

Compulsive-like marble buying behavior represented as the total number of marbles 2/3rd buried in lactating females one hour after bromocriptine (n=11), vehicle (n=10) or L368-899 (n=10) administration. *** ($p<0.001$) and ### ($p<0.001$) indicate significant differences between L368-899 and vehicle or bromocriptine groups, respectively. +++ ($p<0.001$) indicates a significant difference between vehicle and bromocriptine groups. All data is expressed as the mean ± SEM.

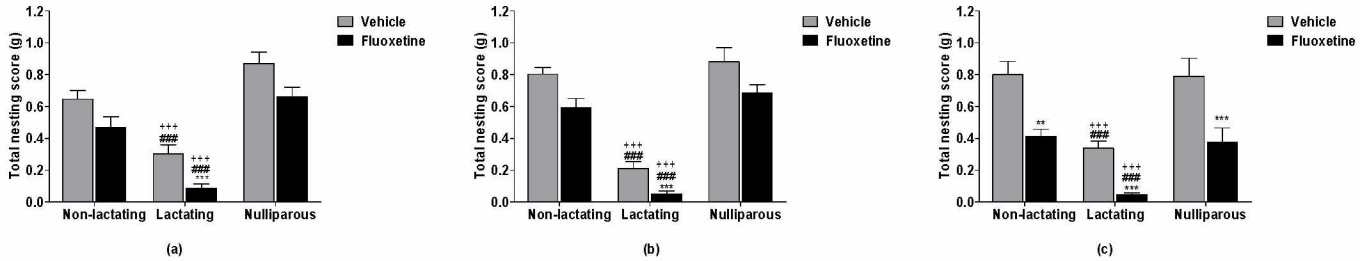

Fig 5.3 Lactating compulsive-like female mice exhibited less compulsive-like nest-building. Compulsive-like nest-building behavior represented as the total nesting score in grams among lactating, non-lactating and nulliparous compulsive-like mice in (a) week 1 (b) week 2 and (c) week 3. ** ($p<0.01$) and *** ($p<0.001$) indicate significant differences between fluoxetine and vehicle groups. ### ($p<0.001$) indicates significant differences between lactating and non-lactating females within each treatment group. +++ ($p<0.001$) indicates significant differences between lactating and nulliparous females within each treatment group. Data is expressed as the mean ± SEM.

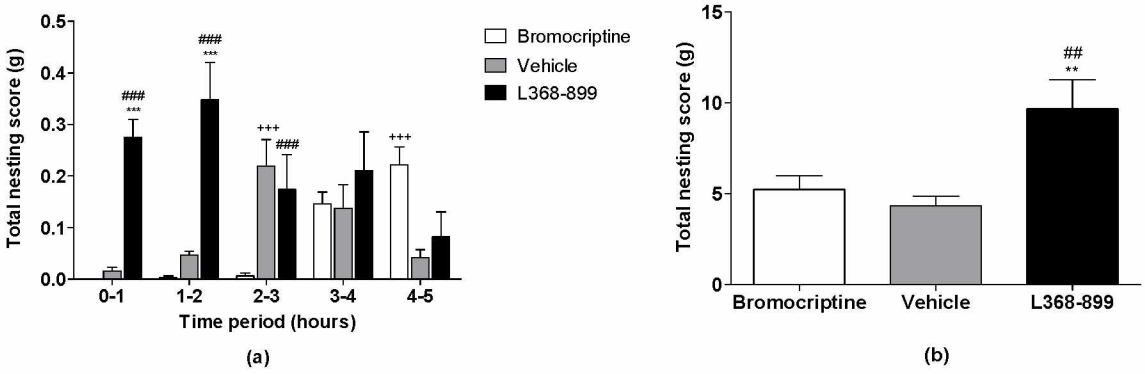

Fig 5.4 The OXTR antagonist increased compulsive-like nest-building in lactating compulsive-like female mice.

Compulsive-like nest-building behavior expressed as total nesting score in grams in lactating compulsive-like female mice (a) 1-5 hours and (b) 0-24 hours after administration with bromocriptine (n=11), vehicle (n=11) or L368-899 (n=12). ** ($p<0.01$) and *** ($p<0.001$) represent significant differences between L368-899 and vehicle groups. ## ($p<0.01$) and ### ($p<0.001$) represent significant differences between L368-899 and bromocriptine groups. +++ ($p<0.001$) represents significant differences between vehicle and bromocriptine groups. All data is expressed as the mean ± SEM.

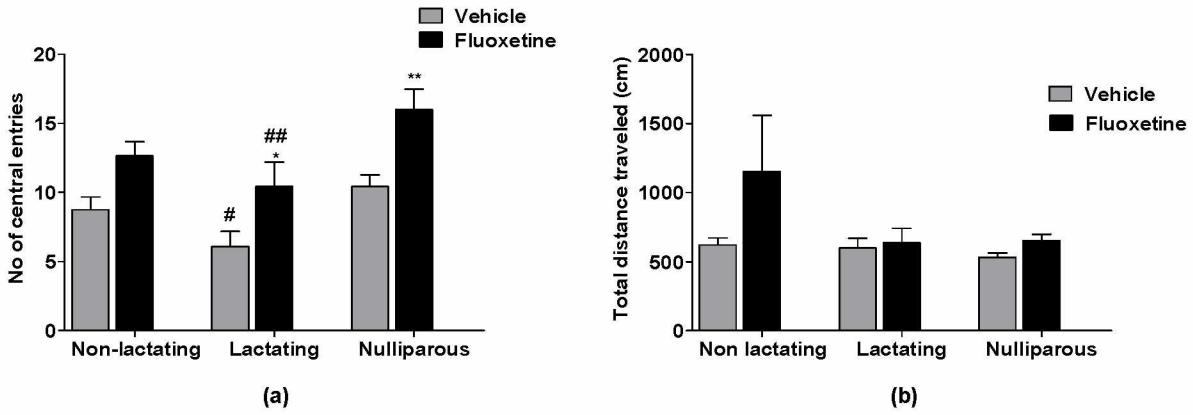

Fig 5.5 Lactating compulsive-like female mice exhibited anxiety-like behavior.

Open field behavior represented as (a) anxiety-like total number of central entries among the lactating, non-lactating and nulliparous females administered with vehicle or fluoxetine. (b) Locomotor activity as the total distance traveled in cm among lactating, non-lactating and nulliparous female mice treated with vehicle or fluoxetine. * ($p<0.05$) and ** ($p<0.01$) indicate significant differences between vehicle and fluoxetine groups. # ($p<0.01$) and ## ($p<0.001$) indicate significant differences between lactating and nulliparous females within each treatment groups. All data is expressed as the mean ± SEM.

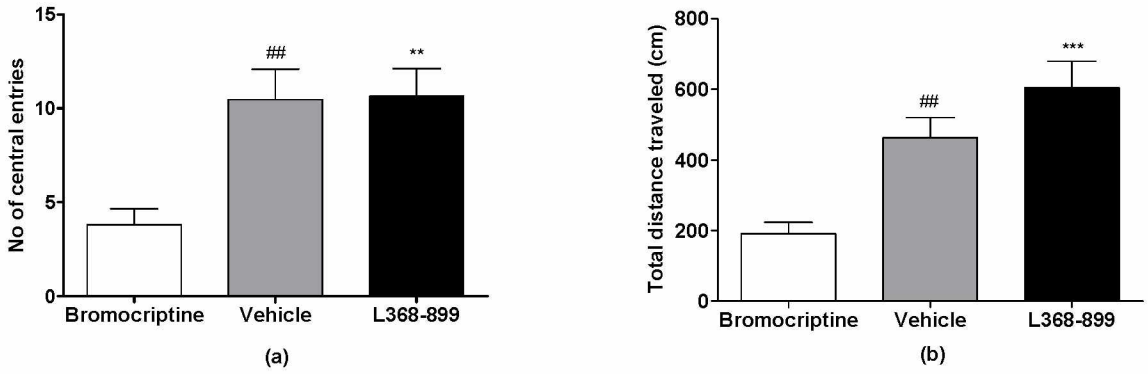

Fig 5.6 The OXTR antagonist had no effect on anxiety-like behavior, while D2R agonist suppressed overall locomotion.

Open field behavior represented as (a) total number of central entries and (b) locomotor activity in lactating females one hour after bromocriptine (n=10), vehicle (n=11) or L368-899 (n=11) administration. ** ($p<0.01$) and *** ($p<0.001$) indicate significant differences between L368-899 and bromocriptine groups. ## ($p<0.01$) indicates significant differences between vehicle and bromocriptine groups.

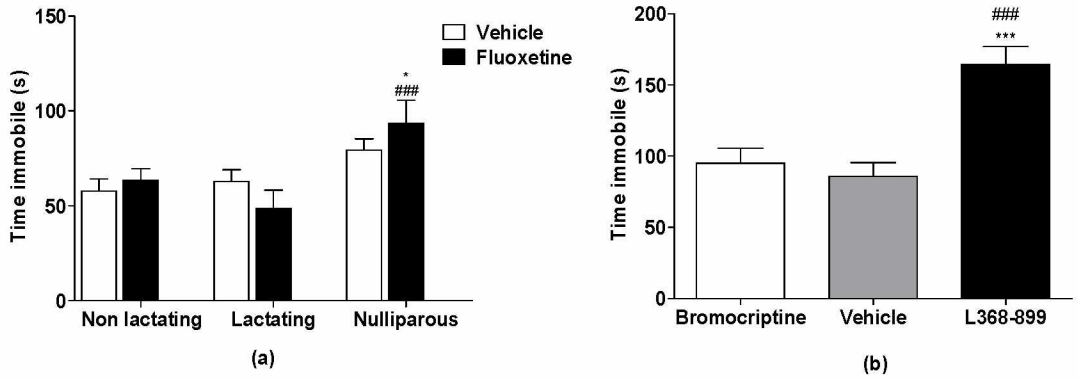

Fig 5.7 Depression-like tail suspension behavior in lactating compulsive-like female mice was increased by the OXTR antagonist.

The depression-like tail suspension test is represented as total immobility time. (a) Lactating, non-lactating and nulliparous females administered with vehicle or fluoxetine. * ($p<0.05$) represents a significant difference between nulliparous and non-lactating females in the fluoxetine group. ### ($p<0.001$) represents a significant difference between nulliparous and lactating females in the fluoxetine group. (b) Lactating females administered with bromocriptine (n=10), vehicle (n=10) or L368-899 (n=10). *** ($p<0.001$) represents a significant difference between L368-899 and vehicle groups. ### ($p<0.001$) represents a significant difference between L368-899 and bromocriptine groups. All data is expressed as the mean ± SEM.

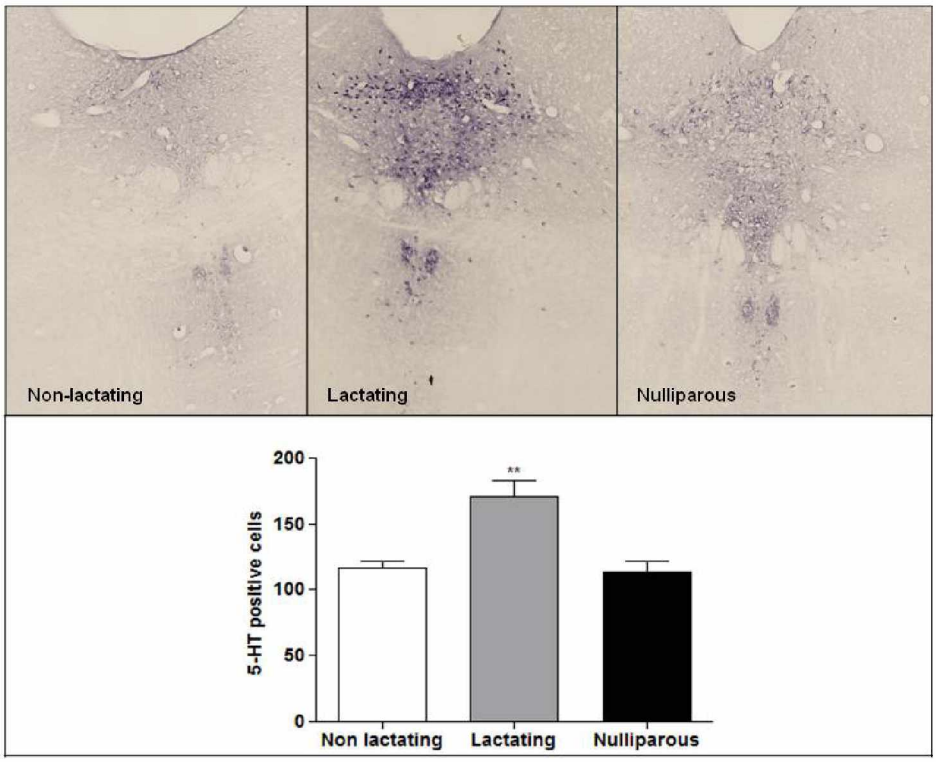

Fig 5.8 Lactating compulsive-like female mice had higher 5-HT immunoreactivity in the dorsal raphe nucleus.

5-HT immunoreactivity represented as 5-HT positive cell count in the DRN of naive lactating (n=5), non-lactating (n=5) and nulliparous (n=5) compulsive-like female mice. ** ($p<0.01$) represents the significant differences of the 5-HT positive cells in lactating females in comparison to both non-lactating and nulliparous females. All data is expressed as the mean ± SEM.

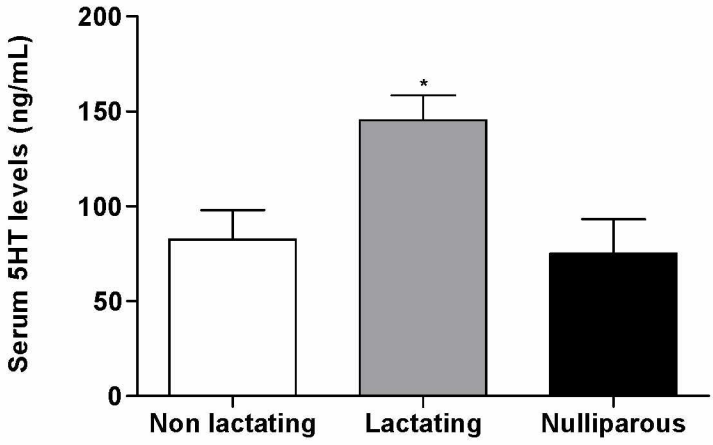

Fig 5.9 Lactating compulsive-like female mice had higher 5-HT levels in serum.

5-HT levels in serum represented as ng/ml in naive lactating (n=6), non-lactating (n=6) and nulliparous (n=6) compulsive-like female mice. * ($p<0.05$) represents the significant differences of the 5-HT serum levels in lactating females in comparison to both non-lactating and nulliparous females. All data is expressed as the mean ± SEM.

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Chapter 6: Attenuation of Compulsive-Like Behavior Through Positive Allosteric Modulation of α4β2 Nicotinic Acetylcholine Receptors in Non-Induced Compulsive-Like Mice.[5]

6.1 Abstract

Nicotinic α4β2 receptors are the most abundant subtypes of nicotinic acetylcholine receptors (nAChRs) expressed in brain regions implicated in obsessive compulsive disorder (OCD). These receptors are known to modify normal and addictive behaviors by modulating neuronal excitability. Desformylflustrabromine (dFBr) is a novel, positive allosteric modulator (PAM) of high acetylcholine sensitivity (HS) and low acetylcholine sensitivity (LS) α4β2 nAChRs. The present study tested the hypothesis that positive allosteric modulation of α4β2 receptors by dFBr will attenuate compulsive-like behavior in a non-induced compulsive-like mouse model. Male mice (Mus musculus) selected for compulsive-like nesting behavior (NB; 48 animals; 12 per group) received acute (once) and chronic (every day for 32 days) subcutaneous injection of dFBr at 2, 4 and 6 mg/kg doses. Saline was used as a control (0 mg/kg). Compulsive-like NB was assessed after 1, 2, 3, 4, 5 and 24 h, while compulsive-like marble burying (MB) and anxiety-like open field (OF) behaviors were performed 2 h after dFBr administration. In the acute administration protocol, dFBr dose dependently attenuated NB and MB. Rapid effects (1–2 h after drug administration) of dFBr on MB and NB were observed for the chronic administration which was in congruence with the acute study. Chronic administration also revealed sustained suppression of NB by dFBr following 5 weeks of treatment. In both the acute and chronic regimen dFBr did not modulate OF behaviors. This research demonstrates the novel role of positive allosteric modulation of α4β2 nicotinic receptors by dFBr as a translational potential for OCD.

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6.2 Introduction

Obsessive-compulsive disorder (OCD) is the fourth most common mental disorder (Pittenger et al., 2005). It has a lifetime prevalence of 2.3% and a 12-month prevalence of 1.2% (Ruscio et al., 2010). Patients suffering from OCD suffer from persistent obsessive thoughts causing distress, and perform compulsive repetitive behaviors to alleviate uncomfortable feelings resulting from obsessions (Diniz et al., 2012). OCD can have disabling effects throughout the patient's lifespan in both males and females (Attiullah et al., 2000).

Obsessions can be thematic, such as fear of contamination, pathological doubt, or need for symmetry/order, or somatic obsessions, like aggression. Repetitive compulsive behaviors involve, washing, seeking, counting, sorting, hoarding and searching (Doron and Moulding, 2009; Ghimire and Goit, 2014; Pauls et al., 2014). Although recently declassified as an anxiety disorder (American Psychiatric Association, 2015), many clinicians conceptualize OCD as a spectrum of related disorders (OCRD) sharing common clinical features of anxiety/fear and worry (Stein and Lochner, 2006; Storch et al., 2008; Fornaro et al., 2009). OCRD encompasses a wide range of diseases which includes somatoform (e.g., Hypochondriasis), impulse control (e.g., Trichotillomania, pathological gambling) and tic disorders (e.g., Tourette's syndrome; Fornaro et al., 2009). Selective serotonin reuptake inhibitors (SSRIs) and cognitive behavioral therapy or their combination are often used as first line treatments. However, a large group of patients remain resistant to treatment either partially or completely (Jenike, 2004; Pittenger et al., 2005).

The cholinergic system in the brain is comprised primarily of nicotinic acetylcholine receptors (nAChRs; Paterson and Nordberg, 2000; Kalamida et al., 2007) and muscarinic acetylcholine receptors (mAChRs; Scarr, 2012; Thiele, 2013), members of the cys-loop superfamily of ligand gated ion channels and G protein-coupled receptors, respectively.

Dysregulation of both mAChRs and nAChRs have been strongly associated with several neurological disorders (Janowsky et al., 1972; Freedman et al., 1995; Warpman and Nordberg, 1995; Breese et al., 2000; Salamone and Zhou, 2000; Perry et al., 2001; Woodruff-Pak and Gould, 2002; Ray et al., 2005; Quik et al., 2007; Scarr, 2012).

The α4β2 nAChR is one of the most prevalent nicotinic subtypes expressed in the brain (McGranahan et al., 2011). The α4β2 subtype is expressed in abundance in the dopamine pathways in the midbrain that influence the drug-induced reward system, mood disorders, stress, movement generation and learning (Wise, 2009; Maskos, 2010). α4β2 nAChRs have also been identified in the striatum, thalamus and cortex (Quik et al., 2013), brain areas implicated in OCD (Pena-Garijo et al., 2010; Fitzgerald et al., 2011). In the striatum, α4β2 receptors have also been shown to modulate GABA and dopamine release (McClure-Begley et al., 2009; Perez et al., 2012). In particular, a subtype of the α4β2 receptor with high sensitivity to acetylcholine (HS α4β2) appears to be involved in striatal dopamine release (Anderson et al., 2009). These studies support a modulatory role of α4β2 receptors in neurotransmitter release in circuits affected in OCD.

Positive allosteric modulators (PAMs) enhance agonist responses via increased agonist potency and/or efficacy. Desformylflustrabromine (dFBr) is a novel PAM capable of potentiating acetylcholine-induced whole cell responses by 370% for the HS and 260% for the low sensitivity (LS) α4β2 receptors with an EC50 of 40 μM and 2.5 μM respectively (Weltzin and Schulte, 2010, 2015). It is currently the only selective PAM for α4β2 receptors capable of potentiating the HS form of the receptor involved in striatal dopamine release. As dFBr increases the efficacy of acetylcholine and does not directly activate receptors, it is postulated that its effect in the synapse would be to enhance acetylcholine mediated transmission. Application of dFBr, unlike application of exogenous agonists, would thus retain the control of synaptic activation via presynaptic release of acetylcholine, albeit with increased stimulation

(Weltzin and Schulte, 2015). Only one in vivo study has been conducted to examine the effect of dFBr potentiation of α4β2 nAChR in an in vivo behavioral model. In this study dFBr was shown to attenuate nicotine self-administration in rats (Liu, 2013). The use of HS α4β2 receptors PAMs for the treatment of OCD has not been previously proposed or tested in any animal model. The aim of the current study was to evaluate our hypothesis that acute and chronic administration of dFBr, a novel PAM specific for α4β2 nAChRs and active at the HS α4β2 subtype, will attenuate compulsive-like and anxiety-like behaviors in our non-induced compulsive-like mouse model.

There are few animal models that exhibit consistent and spontaneous differences in compulsive-like behaviors. We have previously shown that our mice exhibit face and predictive validity as a spontaneous non-induced model for OCD-like behaviors (Greene-Schloesser et al., 2011). The current model was achieved by bidirectionally selecting house mice, Mus musculus, for nest-building behavior for 56 generations (Lynch, 1980; Bult and Lynch, 2000). The stock population for the original selection experiment (Lynch, 1980) was a cross among eight inbred strains, i.e., A, AKR, BLB/c, C3H/2, C57BL, DBA/2, Is/Bi and RIII, to yield the HS/Ibg outbred strain (McClearn Ge and Meredith, 1970; Lynch, 1980). Bidirectional selection resulted in three levels of nesting behavior (NB). All BIG mice exhibit consistent excessive NB engaging in rapid and repetitive pulling of cotton through the cage top metal bars amounting to 6–7 g of cotton on an average in 24 h when compared to normal NB (no significant hyperactivity and repetitiveness when introduced to cotton averaging around 0.50–0.70 g in 24 h) by the Control strain (non-compulsive) and very little NB (most of them do not indulge in nesting) by the SMALL strain (non-compulsive). The Control mice therefore serve as a selection control with intermediate levels between compulsive-like BIG and non-compulsive SMALL strains (Bult and Lynch, 2000). NB is homologous to hoarding in humans with OCD (Warneke, 1993), which is considered to be a measure of compulsive-like phenotype in mice (Greene-Schloesser et

al., 2011; Wolmarans De et al., 2016). The BIG mice also uniformly display repetitive marble burying (MB) behavior burying on an average 19–20 marbles. Both these behaviors are significantly attenuated by SSRIs (e.g., fluoxetine) used to treat OCD but not with normal antidepressants (e.g., desipramine; Greene-Schloesser et al., 2011) substantiating the face and predictive validity of the NB and MB phenotype of the BIG mice for investigating compulsive disorders. Hence in the current context of investigation compulsive-like BIG mice have been considered.

6.3 Materials and Methods

6.3.1 Animals

Compulsive-like BIG male mice, Mus musculus, were raised on wood shavings in polypropylene cages (27 cm × 17 cm × 12 cm) under controlled temperature (22 ± 1°C) and light (12:12 light-dark cycle) with free access to food (Purina Mills, Lab Diet Mouse Diet #5015, St. Louis, MO, USA) and water. Animals were 60 days of age at the start of the experiment. The University of Alaska Fairbanks Institutional Animal Care and Use Committee approved the animal care and experimental procedures (protocol # 675023).

6.3.2 Drug Administration

Deformylflustrabromine hydrochloride (dFBr; Abcam Biochemicals) was dissolved in physiological saline (pH = 6.7) to yield final doses of 2 mg/kg (0.27 mg/mL), 4 mg/kg (0.53 mg/mL) and 6 mg/kg (0.80 mg/mL). Saline was used as a vehicle control (0 mg/kg). A mouse of 40 g received an injection volume of 0.3 mL. Injection volumes were proportionally adjusted according to the body weight of individual animals. All behaviors were performed in the light phase of the light:dark cycle. All data were recorded by an individual blinded to the study.

6.3.3 Acute Study

Male BIG mice were divided into four treatment groups comprising vehicle (sterile saline), 2 mg/kg, 4 mg/kg and 6 mg/kg. Animals in each group (n = 12 per group) were tested for nesting on day 1, MB on day 3 and open field (OF) on day 5. On the first day of testing animals randomly received dFBr or vehicle subcutaneously and in subsequent tests received the same dose. Days 2 and 4 were employed to avoid any residual effects of dFBr from previous administration. For nesting, data were collected after 1, 2, 3, 4, 5 and 24 h due to the progressive nature of the NB (The BIG mice typically get excited and indulge in excessive and repetitive NB when introduced to cotton for the first 3–4 h in the light cycle. This excessive and repetitive nesting activity resumes again in the dark cycle). MB and OF behavior was performed 2 h after dFBr administration (Figure 6.1).

6.3.4 Chronic Study

Since the foundation of our animal model was established through effective reversal of compulsive-like NB and MB behaviors by chronic fluoxetine treatment (Greene-Schloesser et al., 2011) we also conducted a chronic regimen to establish the sustained and long term effects of dFBr on NB and MB. Animals belonging to 0, 2, 4 and 6 mg/kg dose group (n = 12 per group) received single subcutaneous injection of dFBr or saline daily for 32 days. NB, MB and OF behaviors were assessed in the final week (weeks 5) after dFBr administration (NB after 1, 2, 3 4, 5 and 24 h and MB after 2 h of drug injection). NB was performed on day 30, MB on day 31 and OF on day 32 (Figure 1).

The dosages and route of administration was determined based on a prior in vivo study of dFBr on rats (Liu, 2013). Studies on rats have shown that dFBr penetrates the blood-brain barrier and reaches the brain amounting to around 36% in the cerebrospinal fluid after 90 min of subcutaneous administration (Liu, 2013).

6.3.5 Assessment of Compulsive-Like Behaviors

6.3.5.1 Nest-Building Behavior

Nest-building behavior (NB) was performed to assess compulsive-like behavior in the mice (Greene-Schloesser et al., 2011). For both the acute and chronic study, compulsive-like male mice were singly housed and provided with a pre-weighed roll of cotton (Mountain Mist cotton batting, Troy, Inc., Chicago, IL, USA) in the cage-top food hopper immediately following subcutaneous injection of dFBr. The cotton roll was weighed after 1, 2, 3, 4, 5 and 24 h. NB was quantified by the grams of cotton used during each testing period (Bult and Lynch, 1996, 1997, 2000; Greene-Schloesser et al., 2011).

6.3.5.2 Marble Burying Behavior

The MB test is an effective test for determining compulsive-like behavior in mice (Takeuchi et al., 2002; Thomas et al., 2009; Angoa-Pérez et al., 2013). Mice generally do not interact with the marbles and therefore the MB test measures only digging behavior (personal observations). Two hours after dFBr administration, compulsive-like male mice were individually introduced to a polypropylene cage (37 cm × 21 cm × 14 cm) containing 20 glass marbles (10 mm in diameter) evenly spaced on 5 cm deep bedding comprised of wood shavings without access to food or water for 20 min (Greene-Schloesser et al., 2011). Testing was carried out in the testing room separate from the housing room. The total number of marbles buried at least 2/3 in the 20-min period was quantified as compulsive-like digging behavior. After the 20-min test, the animals were returned to their home cages.6.2.6 Assessment of Locomotory and Anxiety-Like Behavior.

6.3.6 Assessment of Locomotory and Anxiety-Like Behavior

6.3.6.1 Open Field Test

Anxiety-like behaviors were determined in the OF test (Simon et al., 1994; Prut and Belzung, 2003). Compulsive-like male mice were individually introduced into an OF (40 cm × 40 cm × 35 cm) with a central zone (20 cm × 20 cm). The apparatus was placed underneath an overhead light illuminating the entire OF (Greene-Schloesser et al., 2011). The animals were placed in the center of the OF and their behavior was video taped for 3 min and analyzed with the aid of ANYMazeTM video tracking software (Stoelting Co., Wood Dale, IL, USA). The time spent in the center (anxiety-like measure) and total distance traveled (locomotion) in the entire OF were measured. The OF was cleaned before each test. Prior experiments (Greene-Schloesser et al., 2011) with the BIG mice in OF indicate that a 3 min duration provides consistent outcomes for assessment of locomotory and anxiety-like behaviors and therefore considered for the current experiment.

6.3.7 Statistical Analysis

Statistical analysis was performed in Graphpad Prism (GraphPad Software, Inc.) and Statistical Analysis System Software (SAS Version 9.4, Cary, NC, USA). NB (grams of cotton), MB (number of marbles at least 2/3 buried) and OF measures (time in center and total distance traveled) were expressed as the mean ± standard error of the mean (SEM). The NB data were shown in figures as grams of cotton used, whereas the statistical analysis was conducted on the square-root transformed nesting scores in order to normalize the data (Bult and Lynch, 2000). Nesting scores at different time points, MB and OF results were analyzed by one-way analysis of variance (ANOVA) whereas, overall drug and drug by time interaction effect between 0 h and 5 h was done by two-way repeated ANOVA. Pairwise comparisons for significant differences between doses were tested by the post hoc Bonferroni multiple comparison test. A probability level of $p < 0.05$ was used as an index of statistical significance in all cases.

6.4 Results

6.4.1 dFBr Attenuates Compulsive-Like NB

6.4.1.1 Significant Suppression of Compulsive-Like NB During the First 5 h of Acute dFBr Administration (Figure 6.2A)

There was an overall significant drug ($F_{(3,220)} = 38.60$, $p < 0.0001$) effect during the first 5 h, a significant time ($F_{(4,220)} = 44.71$, $p < 0.0001$) and drug by time interaction effect ($F_{(12,220)} = 7.08$, $p < 0.0001$) in the compulsive-like NB.

Following a 1 h of dFBr administration, there was no significant attenuation of nesting ($F_{(3,44)} = 1.276$, not significant (NS)). Between 1 and 2 h, dFBr administration resulted in dose-dependent and significant reductions in nesting scores ($F_{(3,44)} = 26.42$, $p < 0.0001$). Post hoc assessment revealed that 4 mg/kg ($t_{22} = 6.210$, $p < 0.001$) and 6 mg/kg ($t_{22} = 6.638$, $p < 0.001$) doses of dFBr significantly attenuated NB as compared to the control (saline). This effect was sustained only by 6 mg/kg dose ($t_{22} = 3.727$, $p < 0.01$) between 2 and 3 h ($F_{(3,44)} = 7.906$, $p < 0.0005$) and both 4 and 6 mg/kg ($t_{22} = 3.305$, $p < 0.05$ and $t22 = 4.585$, $p < 0.001$ respectively) between 3 and 4 h ($F_{(3,44)} = 8.094$, $p < 0.0005$). Between 4 and 5 h after dFBr administration, nesting scores were not significantly different ($F_{(3,44)} = 2.375$, NS).

6.4.1.2 dFBr has an Overall Effect on NB Between 0 h and 24 h in the Acute Administration (Figure 6.3A)

Twenty four hours (time 0 through 24 h) after dFBr administration, overall nesting scores were dose-dependently and significantly reduced ($F_{(3,44)} = 7.645$, $p < 0.001$) with the 2 mg/kg ($t_{22} = 6.213$, $p < 0.001$), 4 mg/kg ($t_{22} = 9.774$, $p < 0.001$) and 6 mg/kg ($t_{22} = 10.50$, $p < 0.001$) groups significantly below the control group.

6.4.1.3 dFBr has an Overall Effect on NB During the First 5 h in the Chronic Administration (Figure 6.2B)

Significant drug ($F_{(3,220)} = 4.87$, $p < 0.01$) time ($F_{(4,220)} = 177.12$, $p < 0.0001$) and drug by time interaction effect ($F_{(12,220)} = 2.29$, $p < 0.01$) was observed in the first 5 h of the chronic administration.

Between 0 h and 1 h there was an overall suppression of NB ($F_{(3,44)} = 6.52$, $p < 0.01$) with 4 mg/kg and 6 mg/kg being the most effective doses ($t_{22} = 6.097$, $p < 0.001$ and $t_{22} = 5.394$, $p < 0.001$ respectively). For 1–2 h the NB declined ($F_{(3,44)} = 4.86$, $p < 0.01$) significantly with 2 and 6 mg/kg showing the main attenuating effects ($t_{22} = 3.086$, $p < 0.05$ and $t_{22} = 3.210$, $p < 0.01$ respectively). No significant effect was observed for NB between 2–3 ($F_{(3,44)} = 1.54$, NS), 3–4 ($F_{(3,44)} = 1.01$, NS) and 4–5 ($F_{(3,44)} = 6.52$, NS) h.

6.4.1.4 dFBr has an Overall Effect on NB Between 0 h and 24 h in the Chronic Administration (Figure 6.3B)

Twenty four hours (time 0 through 24 h) after dFBr administration, overall nesting scores were dose-dependently and significantly reduced ($F_{(3,44)} = 8.85$, $p < 0.0001$) with the 2 mg/kg ($t_{22} = 4.574$, $p < 0.05$), 4 mg/kg ($t_{22} = 7.149$, $p < 0.001$) and 6 mg/kg ($t_{22} = 4.555$, $p < 0.05$) groups significantly below the control group.

6.4.2 dFBr Attenuates Compulsive-Like MB Behavior (Figure 6.4)

6.4.2.1 Acute Administration (Figure 6.4A)

MB behavior were significantly reduced ($F_{(3,44)} = 64.62$, $p < 0.0001$) 2 h after dFBr administration. The 2 mg/kg, 4 mg/kg and 6 mg/kg doses decreased MB dose-dependently compared to the control ($t_{22} = 3.428$, $p < 0.01$; $t_{22} = 12.85$, $p < 0.001$; $t_{22} = 7.667$, $p < 0.001$, respectively). The 4 mg/kg and 6 mg/kg doses also attenuated MB behavior more than the 2 mg/kg dose ($t_{22} = 9.426$, $p < 0.001$ and $t_{22} = 5.332$, $p < 0.001$, respectively).

6.4.2.1 Chronic Administration (Figure 6.4B)

dFBr suppressed MB behavior significantly ($F_{(3,44)}$ = 40.03, $p < 0.0001$) in the fifth week of administration. The most effective doses were 4 mg/kg and 6 mg/kg which showed the maximum suppression of MB when compared to control (t_{22} = 8.643, $p < 0.001$; t_{22} = 8.554, $p < 0.001$, respectively). The 4 and 6 mg/kg doses were also significantly lower than the 2 mg/kg dose (t_{22} = 7.039, $p < 0.001$; t_{22} = 6.950, $p < 0.001$, respectively).

6.4.3 dFBr has no Effect on Anxiety-Like OF Behavior (Figure 6.5)

6.4.3.1 Acute Administration

The total distance traveled which is used to quantify locomotor activity was not different among the treatment groups ($F_{(3,44)}$ = 1.213, NS; Figure 6.5A). No significant differences were also observed among the treatment groups for the time spent in center of the OF ($F_{(3,44)}$ = 0.9849, NS; Figure 6.5C).

6.4.3.2 Chronic Administration

For the chronic regimen the total distance ($F_{(3,44)}$ = 0.30, NS) and time in center ($F_{(3,44)}$ = 0.18, NS) did not differ among treatment groups (Figures 6.5B,D).

6.5 Discussion

Evidence exists for cholinergic involvement in OCD (Lucey et al., 1993; Yankelevitch-Yahav and Joel, 2013). Some studies have indicated exacerbation of OCD symptoms induced by nicotine (Abramovitch et al., 2015). In contrast to the higher rates of smoking in patients with psychiatric disorders, such as schizophrenia, bipolar disorder and ADHD, OCD patients report less smoking behavior (Bejerot and Humble, 1999; Bejerot et al., 2000; McCabe et al., 2004; Abramovitch et al., 2014). It has been suggested that nicotinic activation of an already hyperactivated fronto-striatal circuit worsens OCD symptoms (Abramovitch et al., 2015). However, other studies have shown that nicotine augmentation improves clinical symptoms in

patients with OCD (Carlsson, 2001; Pasquini et al., 2005). Glutamatergic hyperactivity associated with OCD may also be due to mediation of glutamate release by nicotinic receptor activation. (Araki et al., 2002; Mansvelder et al., 2002; Pasquini et al., 2005). Studies investigating cholinergic involvement in glutamatergic hyperactivation suggest that nicotine promotes glutamatergic transmission and stabilizes hyperactivity of the neural circuit that originates in the orbitofrontal cortex and projects to the cingulate gyrus, the striatum and the thalamus (Pasquini et al., 2005). PET and fMRI studies in OCD subjects have shown elevated cerebral blood flow, metabolism and activation (indicators of hyperactivity) in the orbitofrontal cortex and amygdala in OCD (Busatto et al., 2000; Carlsson, 2000; Menzies et al., 2008). These regions receive substantial cholinergic innervations (Mesulam et al., 1986; Carlsson, 2000). Based on these prior studies, we investigated the modulatory role of α4β2 nAChRs in compulsive-like and anxiety-like behaviors in the compulsive-like mice model.

Administration of the novel α4β2 PAM, dFBr produced a reduction in compulsive-like NB and MB, but did not alter anxiety-like and locomotor activity in the OF for the acute study. A very similar response to chronic dFBr was observed where the treatment groups showed rapid suppression of NB (1 h and 2 h) and MB (2 h) after dFBr administration. OF behaviors however remained unaffected by the chronic treatment. These results indicate an apparent selectivity of dFBr for compulsive-like behaviors corroborating the hypothesis that potentiation of α4β2 nAChRs could be an alternative approach for suppressing compulsive-like phenotype thereby posing significant translational potential.

In the acute administration, 4 mg/kg and 6 mg/kg dFBr doses had the largest attenuating effects on NB 2 h after injection, while for the chronic administration the suppression effects on NB was visible after the first hour and endured in the second hour with 6 mg/kg showing a more consistent effect. Interestingly, an earlier effect of dFBr (1 h after administration) on NB was observed for the chronic study indicating potential sensitization to dFBr due to repeated

treatment. The attenuating effects gradually decreased during the next 4 h for both the treatment, showing that dFBr had a rapid effect. This result is consistent with the finding that peak levels of dFBr in the cerebrospinal fluid occur 90 min after administration in rats (Liu, 2013). The 2 mg/kg dFBr dose had no immediate attenuating effect on NB. A long term effect of this dose was however seen in both acute (after 24 h) and chronic (week 5) administration indicating that this dose was effective over a longer time period.

The effects of dFBr, 2 h after injection on MB behavior were generally similar to the effects on NB. However, at the 2 h time point in the acute treatment 2 mg/kg moderately and significantly reduced MB behavior. This effect was not significant in the chronic regimen. No significant effect was observed on NB at the same dose and time point in the acute study but had an effect in the chronic study. These different effects of dFBr treatment may indicate subtle differences in the brain mechanisms that control NB and MB behavior. Clinical studies have shown that some OCD patients with specific types of symptoms do not respond to first line therapies in a similar way (McKay et al., 2004). The doses that act to attenuate obsessions and compulsions in general OCD patients typically fail to produce results in treatment resistant ones (Albert et al., 2013). Moreover, recommended doses for first line treatments might vary depending on the severity of the disorder, co-morbid symptoms like anxiety and potential side effects (Hanna et al., 2011; Albert et al., 2013). Though, a common agreement on OCD subtypes is lacking, therapeutic response and results for each OCD subtype are different (Alonso et al., 2001). For example, fluoxetine, a common OCD drug has greater efficacy in washers and obsessive thoughts when compared to checkers (Farnam et al., 2008). Therefore, the variation in dose response to dFBr of compulsive-like MB and NB behavior adds additional heterogeneity to the BIG mouse for assessing drug effects on various compulsive-like phenotypes.

Acute and chronic dFBr regimen failed to modulate anxiety-like (time spent in center) and locomotor (total distance traveled) behaviors in the OF test. Previous studies using the BIG mice have shown a similar effect of SSRIs like fluoxetine, which failed to reduce overall wheel-running locomotion in the compulsive-like BIG mice but significantly attenuated NB and MB behavior (Greene-Schloesser et al., 2011). Separate brain regions and signaling pathways influencing compulsive-like and anxiety-like symptoms are most likely the explanation for the observed lack of a dFBr effect in the OF test. Anxiety is attributed primarily to the amygdala and ventral hippocampus (McHugh et al., 2004), whereas compulsions and obsessions have been linked to dorsolateral prefrontal cortex (Hirosawa et al., 2013), anterior cingulate cortex (Fitzgerald et al., 2005), orbitofrontal cortex (Beucke et al., 2013) and dysregulation of the corticostriatal-thalamo-cortical circuitry (CSTC; Ting and Feng, 2011). These regions receive projections from the amygdala and hippocampus (McDonald, 1991; Eblen and Graybiel, 1995; Welch et al., 2007; Toyoda et al., 2011; Chen and Etkin, 2013) explaining the co-existence of anxiety along with OCD, which appears to be specific to anxiety related to compulsive-like behaviors rather than more generalized anxiety.

Removal or inhibition by antagonists of α4β2 nAChRs abolishes the anxiolytic effects of nicotine, while stimulating these nAChRs receptors with an agonist decreases anxiety-like behavior. In contrast, anxiogenic effects of nicotine withdrawal are enhanced by stimulation of α7 nAChRs and decreased by inhibition of these nAChRs receptors (Kutlu and Gould, 2015). Allosteric modulation of α4β2 nAChRs by dFBr did not affect anxiety-like behavior in the OF test in the BIG mice, suggesting that these nAChRs receptors may not be involved in the control of anxiety in nicotine-naïve mice. A partial agonist of α4β2 nAChRs (ABT-089) caused anxiogenic effects in nicotine-naïve mice (Yohn et al., 2014). Whether this result contradicts our findings or could be due to low affinity of ABT-089 for α7 nAChRs remains to be determined.

In summary, both acute and chronic dFBr was effective in reversing compulsive-like NB and MB, without exerting any influence on anxiety-like and locomotory behaviors. This indicates the therapeutic potential of modulation of α4β2 nAChRs by dFBr for compulsive phenotypes. Due to the rapid rate of onset (a few hours) of the attenuating effects of dFBr on compulsive-like behaviors, this class of specific nicotinic subtype modulators might also provide more immediate suppression effects thereby provide a bridging option to other first line therapies (e.g., SSRIs) that display longer time courses for onset of effectiveness. dFBr maintained its attenuating effects on NB and MB during chronic treatment, and may therefore also represent a novel first line treatment. However, the cellular mechanisms leading to such acute and chronic suppression of compulsive-like behavior and the role of upstream and downstream targets that ultimately modulate phenotypic expression of the behaviors remains to be elucidated. It also remains to be determined if this effect of dFBr is consistent across all rodent models of compulsive-like phenotype. The current study thereby provides a strong impetus for further exploration of these factors in otherwise sparsely explored area of the role of nAChRs in OCD.

6.6 Acknowledgements

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6.7 Figures

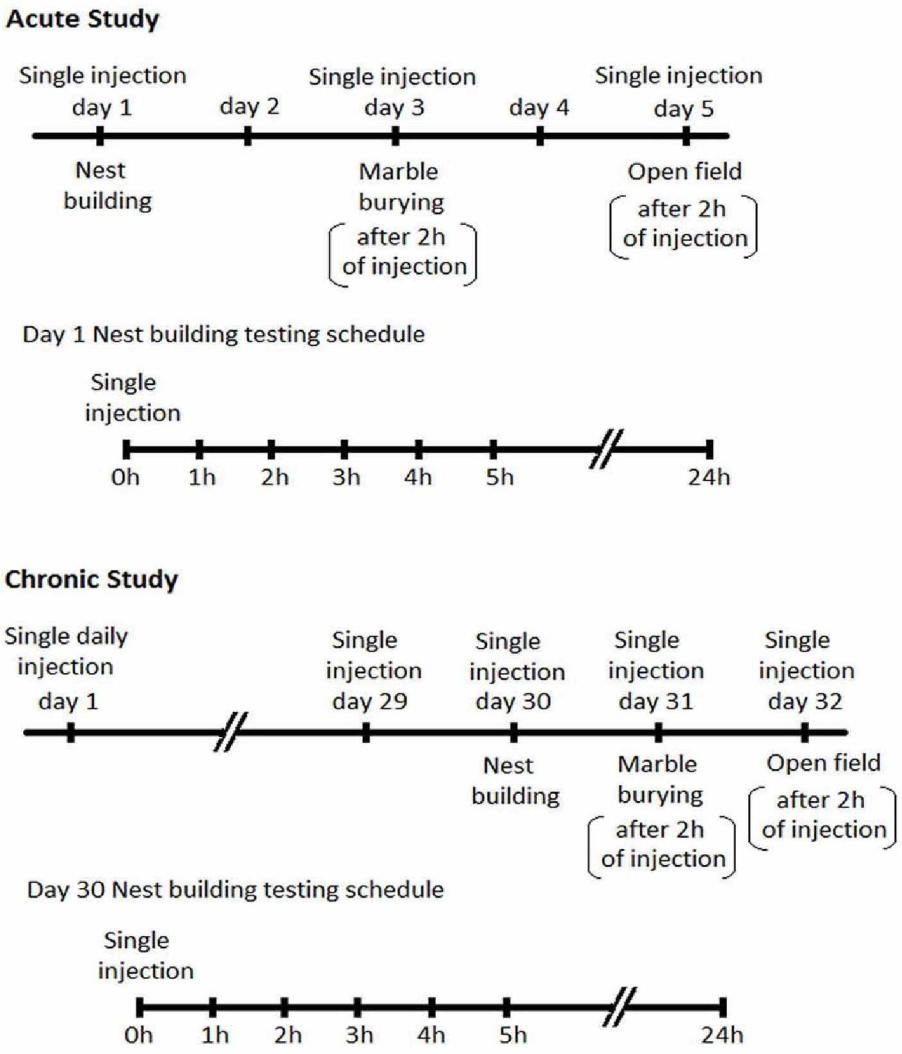

Fig 6.1 Schedules for behavioral assessments following Desformylflustrabromine (dFBr) administration.

6.1 continued: Top panel-Acute Study: mice in all experimental groups (0, 2, 4 and 6 mg/kg) received subcutaneous administration of vehicle or dFBr on days 1, 3 and 5. On day 1, immediately after injections all mice were subjected to nest-building and data were collected 1, 2, 3, 4, 5 and 24 h after injection (nest building testing schedule). On day 3 and 5 all mice were subjected to marble burying (MB) and open field (OF) behaviors, respectively, 2 h after vehicle or dFBr injections. On days 2 and 4 mice were not given injections and were not tested. Lower panel-Chronic study: for the chronic study mice from all groups (0, 2, 4 and 6 mg/kg) received daily single subcutaneous injections of vehicle or dFBr for 32 days. On day 30, immediately after injection all mice were subjected to nest-building and data were collected after 1, 2, 3, 4, 5 and 24 h after injection (nest building testing schedule). On day 31 and 32 all mice were subjected to MB and OF behaviors, respectively, 2 h after vehicle or dFBr injections.

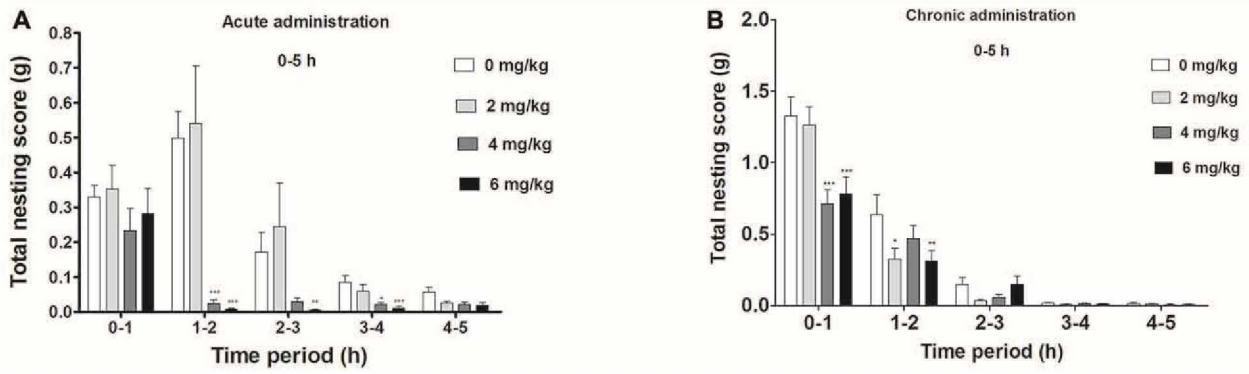

Fig 6.2 Dose-dependent effect of dFBr on compulsive-like NB behavior in compulsive-like BIG mice (n = 12 in each group) from 1–5 h of (A) acute and (B) chronic dFBr administration. Data are expressed as the mean ± standard error of the mean (SEM) for the amount of cotton used in grams. Statistical significance is considered as *p < 0.05, **p < 0.01 ***p < 0.001. All comparisons are with respect to control (saline).

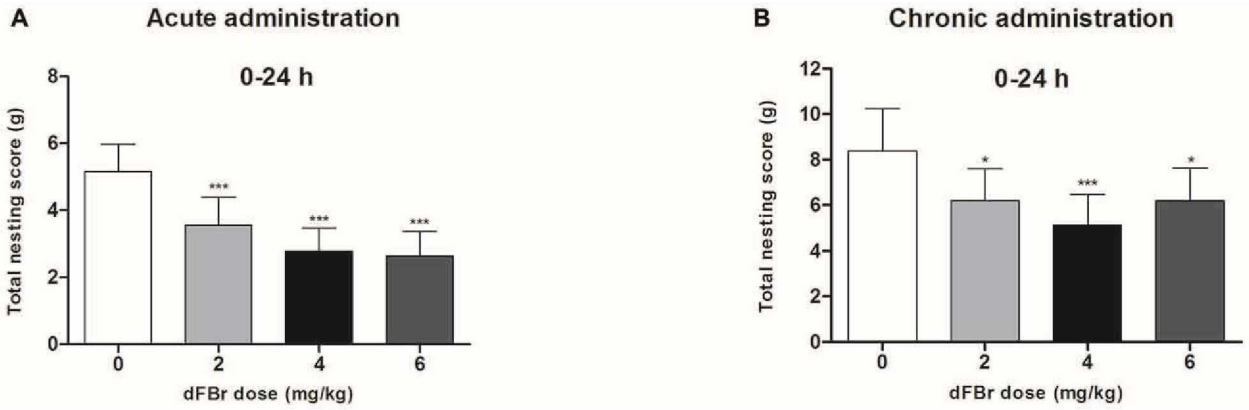

Fig 6.3 Dose-dependent effects of dFBr on overall compulsive-like NB behavior in compulsive-like BIG mice (n = 12 in each group) 0–24 h after (A) acute and (B) chronic dFBr administration. Data are expressed as the mean ± SEM for the amount of cotton used in grams. Statistical significance is considered as *p < 0.05 and ***p < 0.001. All comparisons are with respect to control (saline)

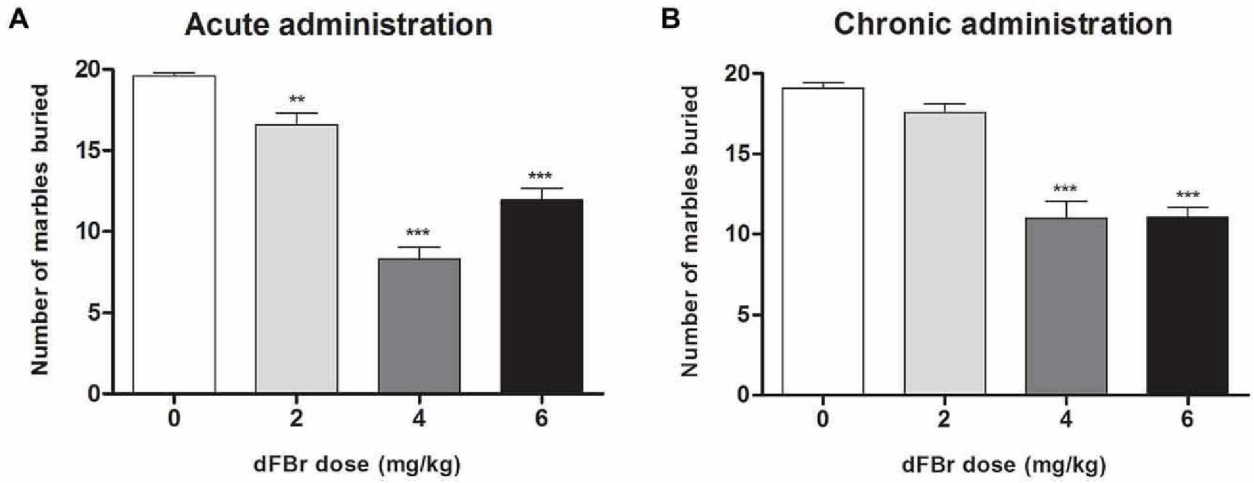

Fig 6.4 Dose-dependent effect of dFBr on compulsive-like MB behavior in compulsive-like BIG mice (n = 12 in each group) 2 h after (A) acute and (B) chronic dFBr administration.

Data are expressed as the mean ± SEM for the number of marbles that are 2/3 buried. Statistical significance is considered as **p < 0.01 and ***p < 0.001. All comparisons are with respect to control (saline).

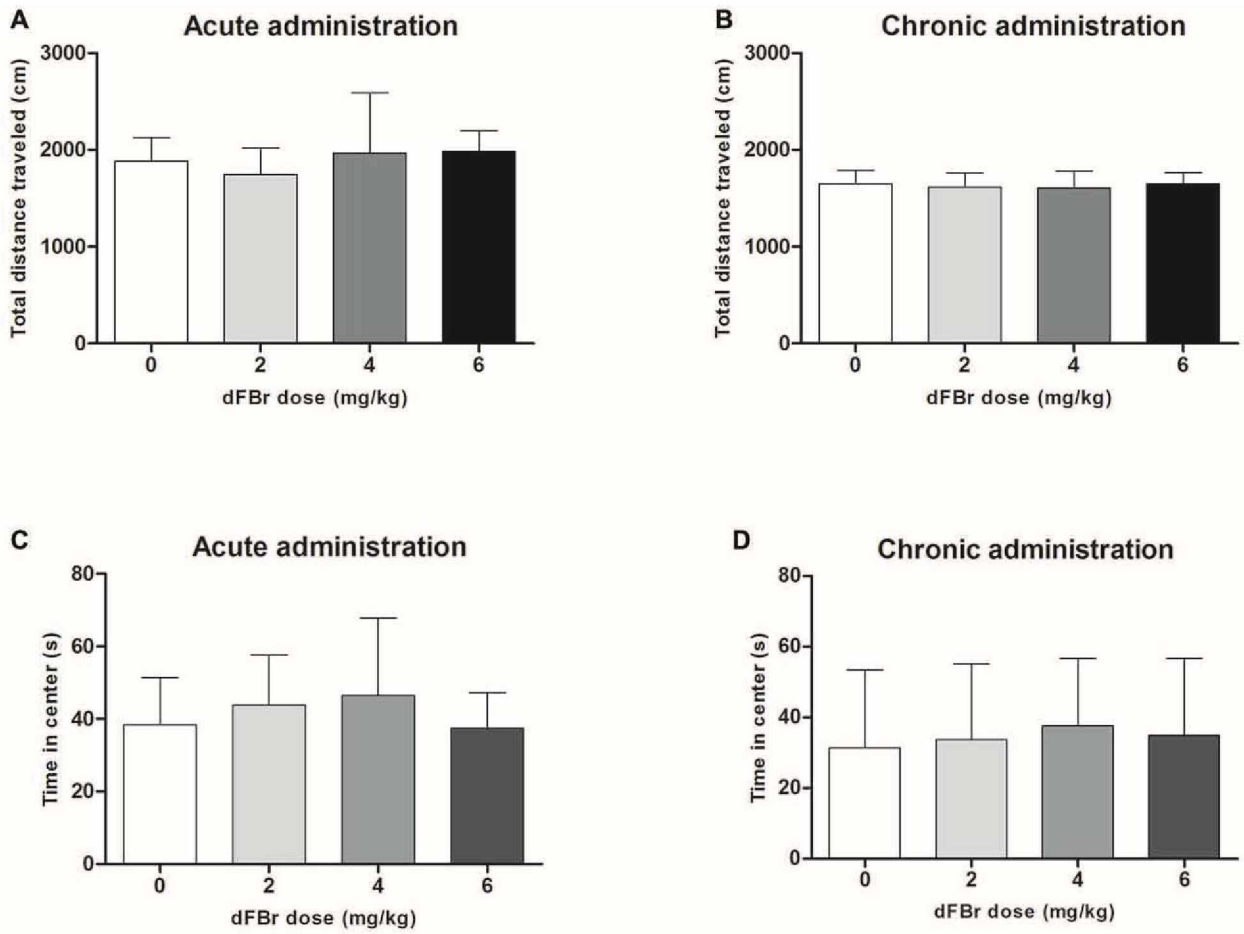

Fig 6.5 Effect of dFBr on OF locomotory activity in (A) acute administration and (B) chronic administration. Anxiety-like time in center in OF in (C) acute administration and (D) chronic administration in compulsive-like BIG mice (n = 12 in each group).

Data are expressed as the mean ± SEM for the total distance traveled in the OF. No statistical significance was found.

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Chapter 7: Conclusion and Future Directions

7.1 General overview

Through this thesis work, I have strengthened the face, predictive and construct validity of a novel mouse model that exhibits a spontaneous compulsive-like phenotype for studying OCD in humans. Due to the complex symptom dimensions of human OCD patients (Leckman et al., 2007) it is impossible to create an animal model that recapitulates all neurobiological substrates and behavioral correlates of human OCD (Joel, 2006). However, investigating the compulsive-like, perseverant and repetitive phenotypes in rodent models can enhance the understanding of the compulsive behavior and its corresponding neural mechanisms in humans (Alonso et al., 2015). Relatively fewer studies have been performed on spontaneous and behaviorally induced models that exhibit phenotypic similarities of repetitiveness and stereotypy (Yadin et al., 1991; Joel and Avisar, 2001; Korff et al., 2008; Greene-Schloesser et al., 2011). Further, the associated anxiety-like, depression-like and cognitive behaviors have been sparsely explored in animal models of OCD (Welch et al., 2007; Shmelkov et al., 2010; Shanahan et al., 2011). The clinical heterogeneity of the disorder due to factors, such as genetics and sex differences (Nestadt et al., 2010; de Mathis et al., 2011; Mas et al., 2014; Mas et al., 2016), also warrants studies that compare compulsive-like strains and investigates sex hormonal influence for face, predictive and construct validity of phenotypic subtypes and associated affective behaviors. Through this research I have attempted to address these factors.

7.2 Interplay of Strain And Sex Modulates Compulsive-Like and Associated Affective Behaviors in Spontaneous Compulsive-Like Mouse Strains.

In the experiments described in chapter 2, I evaluated the compulsive-, anxiety-, depression- and cognitive-like behaviors of our mouse model based on strain and sex differences. Previously, our lab investigated the predictive validity of the mouse model to study OCD (Greene-Schloesser et al., 2011). In this study only males of one of the compulsive-like

and non-compulsive strains were used. The experiments described in chapter 2 were an extension of this study that accounted for males and females of replicate strains of compulsive-like BIG mice (BIG1 and BIG2), randomly bred controls (C1 and C2) and non-compulsive SMALL mice (SML1 and SML2). This comparison of both males and females of all the replicate strains furthered the understanding of the differences in behavioral phenotypes that were influenced by strain and sex. The results from the study indicated that female BIG strains have face validity for studying OCD (Mitra et al., 2017). This was not established before. I also showed that in the proestrus stage of the estrus cycle when estrogen levels are considered to be higher in circulation, the BIG females were less compulsive than males (Mitra et al., 2017). One of the most important findings of this study was that sex by genotype interactions influence behavioral outcomes in the compulsive-like condition. Replicate, strain and sex effects were observed in compulsive-like phenotypes, whereas replicate, strain and sex effects influenced anxiety-like open field, elevated plus maze and depression-like forced swim behaviors, respectively. Strain differences were also observed in the mouse strains for HPA axis response to stress (Mitra et al., 2017). Overall this data indicates that sex and genotype interaction can influence obsessive compulsive symptomology in humans and strengthens the validity of our mouse model for studying such interactions on treatment, behavioral outcomes and neurobiological substrates. This can assist in pinpointing critical neurobiological candidates for understanding clinical heterogeneity in human OCD patients.

As a continuation to this study, it would be interesting to assess the behavioral outcomes in various estrous stages of BIG females, which were not performed in the current study. It would also be interesting to investigate baseline HPA axis response of the strains. A more robust cognitive assessment could be included to look for various forms of memory consolidation such as spatial and long-term memory.

7.3 Predictive Validity of BIG Male Mouse Strains for Evaluation of Clinical Heterogeneity in OCD

Chapter 3 was a follow up of chapter 2, where I investigated the role of genetic background in influencing drug efficacy. Using BIG1 and BIG2 males, I evaluated the strain and compulsive-trait differences in response to fluvoxamine, a common first line treatment option for OCD patients. Chronic administration of fluvoxamine daily for 18 days resulted in a dose-dependent attenuation of compulsive-like nesting behavior for BIG1 male mice one hour after drug administration. Surprisingly, I did not observe any effect of the drug on compulsive-like nesting for BIG2 male mice. This was an interesting finding since fluvoxamine dose-dependently attenuated compulsive-like marble burying behavior in both the BIG1 and BIG2 males. Fluvoxamine treatment also did not affect anxiety-like open field and locomotory behaviors in BIG1 strain, while in BIG2 strain the effect was observed for a medium dose of 10 mg/kg. This data is very interesting since it establishes the predictive validity of our model to study clinical heterogeneity associated with OCD. Clinical studies have shown that some OCD patients with specific symptoms respond to first line treatment better than patients with a different symptom dimension (Farnam et al., 2008). These symptoms categorize patients into OCD subgroups.

Compulsive-like phenotypes in rodents such as nest-building and marble burying is considered to be human equivalent of hoarding (Warneke et al., 1993) and repetitive/perseveration behaviors (Thomas et al., 2009; Angoa-Perez et al., 2013), respectively, as commonly seen in OCD. Hence, these two behaviors can be used to understand two separate compulsive facets in humans. The trait specific response of the BIG strains to fluvoxamine further corroborates this hypothesis. I showed through chapter 2 that though BIG strains were generally less anxious than SMALL strains, within each BIG strain the BIG2 males exhibited more anxiety-like behavior than BIG1 strains (Mitra et al., 2017). The BIG2 strains responded to the fluvoxamine dose of 10 mg/kg for the time spent in center of the open field.

This effect was not seen in other measures of anxiety and locomotion such as line crossings and latency to leave the center. Hence, behavioral responses to fluvoxamine in the BIG strains can be very specific to assessed parameters and type of anxiety-like measure and could vary between strains. Results from chapter 3 therefore provide a foundation for using BIG strains in further exploration of neurobiological mechanisms of drug resistance that is seen in OCD patient subtypes.

For future experimentation inclusion of female BIG strains will be considered. RNA sequencing of brain regions implicated in OCD that have been treated with first line drugs such as, SSRIs in both sexes is also a future direction. This would provide a gene expression profile of candidate genes and would also enable screening of new target genes. Other anxiety-like assessments such as elevated plus maze, light-dark chamber tests could be included to evaluate strain differences and treatment responses to various forms of emotionality associated with compulsive-like behaviors.

7.4 Predictive and Construct Validity of the BIG Compulsive-Like Female Mouse Strains During Surgical Menopause

Through chapter 4, I established the predictive and construct validity of the BIG1 and BIG2 females on the premise of the possible role of ovarian sex steroids in compulsive behaviors. Only few studies have so far evaluated the role of sex hormonal manipulations on compulsive-like phenotypes (Fernandez-guasti et al., 2006; Flaisher-Greenberg et al., 2009). This is critical since it can provide significant understanding of the role of ovarian sex steroids in regulating obsessions and compulsions. My results showed that there was an exacerbation of compulsive-like and anxiety-like behaviors in compulsive-like strains (BIG1 and BIG2) only with a trait specific variation in compulsive-like behavior among BIG1 and BIG2 females. The BIG1 sham and ovariectomized females had higher compulsive-like nesting scores when compared to BIG2 sham and ovariectomized females (Mitra et al., 2016a). This replicate effect was not seen

in the nesting scores once the ovariectomized strains were treated with either 17β-estradiol (E2) or progesterone (P4). The BIG strains (BIG1 and BIG2) however did not exhibit the same replicate effect for compulsive-like marble burying behavior. Acute E2 administration attenuated compulsive-like nesting and marble burying, while P4 administration had no effect on these behaviors. For the anxiety-like measures there was a strain specific response to E2 and P4 administration (Mitra et al., 2016a). Overall this data adheres to the clinical heterogeneity associated with OCD (Fontenelle et al., 2005; Grados and Riddle, 2008; Leckman et al., 2009) and opens future avenues for investigating hormonal therapy for OCD patients undergoing surgical menopause. My results also established the construct validity of the BIG females based on ovarian sex steroids to study neurobiological substrates and concomitant behavioral correlates in OCD during surgical menopause.

A comparison between acute versus chronic ovariectomy in expression of behavioral phenotypes in the BIG strains could be a future direction. Also, acute versus chronic hormone treatment and a combinational treatment (E2+P4) experiment would be essential to understand the scope of hormonal intervention in the compulsive-like condition. This acute versus chronic ovariectomy could further elucidate the behavioral outcomes between surgical versus progressive menopause in compulsive-like mice. It can lead to better understanding of the differential role of surgical and natural menopause in influencing obsessions, compulsions, anxiety, depression and cognition in human OCD patients. Deciphering the role of estrogen and or progesterone receptor subtypes in mediating these behavioral expressions in OCD implicated brain regions would further address the neurobiological mechanisms attributed to these hormone challenge stages in compulsive-like female mice and thereby female OCD patients. The long term goal would be to facilitate tailor made hormone replacement therapy effective for various physiological stages in female OCD patients.

7.5 Postpartum Lactation-Mediated Modulation of Behavioral Outcomes in Compulsive-Like Female Mice is Partly Oxytocin Mediated.

In chapter 5, I investigated the role of postpartum lactation in influencing behavioral expression in the compulsive-like mice. The findings indicated that lactating female compulsive-like mice exhibited less compulsive-like nesting and marble burying when compared to non-lactating and nulliparous females. When treated with chronic fluoxetine the attenuation of compulsive-like behavior was enhanced for lactating females when compared to non-lactating and nulliparous controls for marble burying, but not for nest-building. For anxiety-like behaviors, lactating females showed more anxiogenic behavior in comparison to non-lactating and nulliparous females. Fluoxetine treatment did not produce an enhanced responsiveness to anxiety-like behavior in the non-lactating females. No difference was observed for depression-like tail suspension behavior. The serum levels of serotonin (5-HT) and dorsal raphe nucleus (DRN) 5-HT immunoreactivity were also higher for lactating females. This indicates that there is no balancing effect between peripheral and central serotonin signaling during lactation as proposed in a prior study with wild type C57BL6/J mice (Jury et al., 2015). It could also mean that the serotonin signaling during lactation can vary in normal versus psychiatric condition giving rise to differential behavioral outcomes as seen in our mouse model. This however is not established in my current study and requires further investigation.

My data also showed that the anti-compulsive effect of lactation was eliminated in female mice treated with an oxytocin receptor inhibitor, while suppression of lactation through activation of dopaminergic system reduced the overall locomotion of the animals. This suppression in locomotory function resulted in less compulsive-like behavior, but more anxiety-like behavior indicating that these were most likely not drug specific effects. When assessed in the depression-like tail suspension test however, the dopamine receptor agonist group of lactating female mice showed no difference in immobility. The oxytocin receptor antagonist

group however had higher immobility indicating more depression-like phenotype. Overall, the data indicated the role of oxytocin during postpartum lactation in attenuating compulsive-like and depression-like behaviors. Postpartum is associated with precipitation of mood disorders and pre-existing psychiatric disorders such as OCD during postpartum has been sparsely researched (House et al., 2016; Brandes et al., 2004; Miller et al., 2013). Hence, the current study provides valuable insight into the lactation-mediated behavioral modulation during the postpartum phase. This can be replicated to human OCD patients for effective treatment strategies during postpartum conditions.

Including BIG1 females for strain comparison in lactation-induced behavioral and neurobiological outcomes would be an interesting future research objective to replicate the results in the BIG2 mice. Also, treating lactating compulsive-like females with fluoxetine along with oxytocin receptor antagonist or D2 receptor agonist would be a useful future aim in evaluating mechanism of enhanced responsiveness to SSRIs.

7.6 Allosteric Modulation of Nicotinic Receptor Subtype Can Attenuate Compulsive-Like Phenotypes.

The role of nicotinic receptor subtypes in modulating compulsive-like behavior was explored in the final thesis chapter. The α4β2 subtype of neuronal nicotinic receptors is predominantly expressed in brain regions involved in the drug-induced reward system, mood disorders, stress and learning (Wise, 2009; Maskos, 2010). These receptor subtypes are also expressed in brain regions implicated in OCD (Pena-Garijo et al., 2010; Fitzgerald et al., 2011; Quik et al., 2013). Hence, I investigated the modulatory role of these receptors on expression of compulsive-like and anxiety-like phenotypes in the compulsive-like mouse model. Desformylflustrabromine (dFBr), a novel positive allosteric modulator of α4β2 (Weltzin and Schulte, 2010), was used for this study. The results showed a consistent suppression of compulsive-like nest-building and marble burying behavior in the BIG1 males treated both

acutely and chronically with dFBr. dFBr however did not have any modulatory role on locomotion and anxiety-like behavior in the open field (Mitra et al., 2016b). The findings from this chapter point towards the potential role of nicotinic receptor subtype potentiation in attenuating compulsive-like behaviors. This could have significant translational prospect for treatment of OCD and OCD related (OCRD) disorders.

Future studies would aim at investigating the neurobiological mechanisms attributing to this attenuation of compulsive-like behavior due to dFBr administration.

7.7 Overall Conclusion

Finally, this research does not intend to establish a mouse model that represents all aspects of human OCD. This would be impossible, considering the species specific complexity that happen in human OCD patients based on personal experiences and events. Through this research, I have attempted to understand the complex interplay of sex and strain in influencing neuronal building blocks of behavior in a spontaneously compulsive-like mouse model. This thesis also highlights the importance of ovarian steroids, postpartum lactation and modulation of the cholinergic system in influencing behavioral expression in the compulsive-like mice. This can contribute to the understanding of analogous behaviors in human OCD patients and the variables such as genetics, sex differences and physiological stages that influence the symptomology and neurobiology of the disorder.

7.8 References

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Institutional Animal Care and Use Committee
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August 14, 2013

To: Cheryl Frye, PhD
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [497513-2] Sex, neurohormonal, and genetic differences in a mouse model that diverges based on compulsive-like phenotype.

The IACUC reviewed and approved the Amendment/Modification referenced above by Designated Member Review.

Received:	August 13, 2013
Approval Date:	August 14, 2013
Initial Approval Date:	August 14, 2013
Expiration Date:	August 14, 2014

This action is included on the August 15, 2013 IACUC Agenda.

Abel Bult-Ito must not handle animals until required training is complete.

PI responsibilities:

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May 20, 2015

To:	Abel Bult-Ito Principal Investigator
From:	University of Alaska Fairbanks IACUC
Re:	[731871-4] Research: ASRA Behavioral Neuroscience Research Educational Experience

The IACUC reviewed and approved the Amendment/Modification referenced above by Designated Member Review.

Received:	May 19, 2015
Approval Date:	May 20, 2015
Initial Approval Date:	May 20, 2015
Expiration Date:	May 20, 2016

This action is included on the May 27, 2015 IACUC Agenda.

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March 14, 2014

To: Abel Bult-Ito, PhD
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [568518-2] OCD Estrogen Research Protocol-Abel Lab

The IACUC reviewed and approved the Response/Follow-Up referenced above by Full Committee Review.

Received:	March 9, 2014
Approval Date:	March 13, 2014
Initial Approval Date:	March 13, 2014
Expiration Date:	March 13, 2015

This action is included on the March 13, 2014 IACUC Agenda.

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This action is included on the August 14, 2014 IACUC Agenda.

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July 30, 2014

To: Abel Bult-Ito, PhD
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [631126-3] Acute estrogen administration in hormonal deprived compulsive-like female mice will attenuate compulsive-like behavior and restore cognitive and affective functions.

The IACUC reviewed and approved the modification to the Protocol referenced above by Designated Member Review.

Received:	July 16, 2014
Approval Date:	July 30, 2014
Initial Approval Date:	July 30, 2014
Expiration Date:	July 30, 2015

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February 28, 2017

To: Abel Buit-Ito
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [862663-11] Lactation Project

The IACUC reviewed and approved the Amendment/Modification to the Personnel List referenced above by Administrative Review.

Received:	February 28, 2017
Approval Date:	February 28, 2017
Initial Approval Date:	February 22, 2016
Expiration Date:	February 22, 2018

This action is included on the March 9, 2017 IACUC Agenda.

PI responsibilities:

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December 3, 2014

To: Abel Bult-Ito, PhD
Principal Investigator

From: University of Alaska Fairbanks IACUC

Re: [675023-4] Investigation of desformylflustrabromine (dFBr) as a novel target for attenuation of compulsive-like and anxiety behavior in non-induced compulsive-like mouse.

The IACUC reviewed and approved the Amendment/Modification to the protocol and approval of the personnel list referenced above by Designated Member Review.

Received:	December 2, 2014
Approval Date:	December 3, 2014
Initial Approval Date:	December 3, 2014
Expiration Date:	December 3, 2015

This action is included on the December 11, 2014 IACUC Agenda.

PI responsibilities:

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